

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
20 September 2001 (20.09.2001)

PCT

(10) International Publication Number  
**WO 01/69262 A1**

- (51) International Patent Classification<sup>7</sup>: G01N 33/74, C12N 15/00 (74) Agent: FASSE, J., Peter; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).
- (21) International Application Number: PCT/US01/08276 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date: 15 March 2001 (15.03.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 60/189,605 15 March 2000 (15.03.2000) US (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:  
US 60/189,605 (CON)  
Filed on 15 March 2000 (15.03.2000)
- (71) Applicant (*for all designated States except US*): UNIVERSITY OF MASSACHUSETTS [US/US]; 26th Floor, One Beacon Street, Boston, MA 02108 (US). Published:  
— with international search report  
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- (72) Inventor; and
- (75) Inventor/Applicant (*for US only*): HO, Shuk-Mei [US/US]; 9 Chase Avenue, Lexington, MA 02421 (US). For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ER $\beta$ -MEDIATED GENE EXPRESSION

(57) Abstract: An *in vitro* screening method for identifying a compound that modulates ER $\beta$ -mediated cell growth inhibition is disclosed. The method includes: providing a mammalian test cell containing a functional ER $\beta$  protein; contacting the test cell with a candidate compound; and detecting an increase or decrease in the expression of an ER $\beta$ -regulated gene in the presence of the candidate compound. Compounds that modulate ER $\beta$ -mediated cell growth inhibition can promote or inhibit this process. In some embodiments, the test cell contains no detectable ER $\alpha$  protein. The ER $\beta$ -regulated gene can be, e.g., the genes encoding receptor-like tyrosine kinase (RYK), 5-hydroxytryptamine A1 receptor (E2c), BCL-2 related A1, embryonic growth/differentiation factor, IL-12, TL1309, or IFN- $\alpha/\beta$  receptor.

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## ER $\beta$ -Mediated Gene Expression

This patent application claims priority from provisional application serial no. 60/189,605 filed on March 15, 2000, which is incorporated herein by reference in its entirety.

### FEDERALLY SPONSORED RESEARCH

Work on the invention was supported in part by U.S. Army grant no. DAMD17-98-1-8606. Therefore, the government has certain rights in the invention.

### TECHNICAL FIELD

5 This invention relates to molecular biology, urology, neurobiology, endocrinology, and oncology.

### BACKGROUND

10 Estrogen, a steroid hormone, is involved in growth, differentiation and function of various target tissues in the female and male reproductive systems, including the male prostate gland. Estrogen is also plays certain roles in the central nervous system and the cardiovascular system. Estrogen crosses cell membranes and exerts its effects through binding with high affinity to an estrogen receptor (ER) in the cell nucleus.

Historically, the actions of estrogens/antiestrogens were thought to be mediated by the classical ER, now called ER $\alpha$ . In human and rodent prostates, ER $\alpha$  is localized in the stromal compartment and basal epithelial cells of the prostate (Prins et al., *Endocrinology*, 138:1801-1809, 1997; Bonkhoff et al., *Am. J. Pathol.*, 155:641-647, 1999; Wernert et al., *Virchows Arch. A. Pathol. Anat. Histopathol.* 412:387-391, 1988; Ehara et al., *Prostate*, 27:304-313, 1995; Kirschenbaum et al., *J. Androl.*, 15:528-533, 1994; Hiramatsu et al., *Histopathology*, 28:163-168, 1996). Because ER $\alpha$  is not expressed in the normal glandular epithelium of rat or human prostate (Hartley-Asp et al., *Mutat. Res.*, 143:231-235, 1985; Lau et al., *Endocrinology*, 139:424-427, 1997; Bonkhoff et al., *Am. J. Pathol.* 155:641-647, 1999; Ehara et al., *Prostate*, 27:304-313, 1995; Kirschenbaum et al., *J. Androl.*, 15:528-533, 1994; Hiramatsu et al., *Histopathology*, 28:163-168, 1996; Rohlff et al., *Prostate*, 37:51-59, 1998), it has seemed likely that the action of estrogen/antiestrogen on normal prostate epithelial cells (PrECs) is indirect, likely mediated via estrogen-induced stromal factors.

25

In 1996, the cloning of ER $\beta$ , a second subtype of ER, was reported. ER $\beta$  was found to be expressed at high levels in the epithelial compartments of the rat prostate (Mosselman et al., *FEBS Lett.* 392:49-53, 1996; Kuiper et al., *Proc. Natl. Acad. Sci. USA* 93:5925-5930, 1997). Although ER $\beta$  shares high homology with the classical ER (ER $\alpha$ ), it has been suggested that the two ER subtypes regulate different sets of cellular functions (Paech et al., *Science*, 277:1508-1510, 1997; Montano et al., *J. Biol. Chem.*, 273:25443-25449, 1998.).

Several investigators have reported ER $\alpha$  expression in human prostate cancer cell lines, including LNCaP, PC-3 and DU-145 (Carruba et al., *Cancer Res.*, 54:1190-93, 1994; Castagnetta et al., *Endocrinology*, 136:2309-2319, 1995), but others have reported failure to find such results (Hobisch et al., *J. Pathol.*, 182:356-361, 1998.). Observations on ER $\alpha$  expression in prostate cancer specimens also have conflicted. Bonkhoff and co-workers reported ER $\alpha$  expression to be infrequent in low-to-moderate grade adenocarcinoma, but common in high grade and metastatic cancers (Bonkhoff et al., *supra*).

Similarly, published information concerning the distribution of ER $\beta$  in normal and malignant human PrECs has conflicted. At least one study reported lack of ER $\beta$  expression in human prostate tissues (Bonkhoff et al., *supra*), but other published reports noted expression of this receptor subtype in basal epithelial cells of the human (Lau et al., *Proc. Amer. Assoc. Cancer Res. Annual Meeting*, 40:637-638, 1999; Sinisi et al., *Proc. Endocrine Soc., 81<sup>st</sup> Ann. Mtg*, P1-611, 1999; Taylor et al., *Proc. Endocrine Soc. 81<sup>st</sup> Ann. Mtg*, P1-228, 1999).

In view of such conflicting reports, the roles played by estrogens in the neoplastic transformation of PrEC, and in prostatic carcinoma progression and treatment, have been controversial. Exposure of human or rodent to estrogens induces a proliferative lesion, squamous metaplasia, and in their prostates (Sugimura et al., *Hum. Pathol.*, 19:133-139, 1988; Yonemura et al., *Acta Anat.* (Basel), 153:1-11, 1995; Triche et al., *Lab. Invest.*, 25:596-606, 1971; Levine et al., *J. Urol.*, 146:790-793, 1991), while prolonged treatment of Noble rats with androgen plus estrogen causes a high incidence of prostate adenocarcinoma in the dorsolateral prostates of the treated animals (Noble et al., *Cancer Res.*, 40:3547-3550, 1980; Drago, *Anticancer Res.*, 4:255-256, 1984; Leav et al., *Prostate*, 15:23-40, 1995; Bosland et al., *Carcinogenesis*, 16:1311-1317, 1995). Paradoxically, DES, TAM and other estrogens have been used as treatment regimens for advanced metastatic prostate cancer

(Ahmed et al., *Int. Urol. Nephrol.* 30(2): 159-64, 1998; Bergan et al., *Proc. Amer. Soc. Clin. Oncol.* 14:A637, 1995; Bergan et al., *Clinical Cancer Res.* 5:2366-2373, 1999; Klotz et al., *J. Urol.*, 161:169-172, 1999; Smith et al., *Urology*, 52:257-260 1998). In addition to acting as chemical castration agents, both estrogen and antiestrogen are believed to exert direct growth inhibitory effects on prostatic cancer cells via induction of apoptosis or cell cycle arrest (Brehmer et al., *J. Urology*, 108:890-896, 1972; Hartley-Asp et al., *Mutat. Res.* 143:231-235, 1985; Schulze et al., *Prostate*, 16:331-343, 1990; Robertson et al., *J. Natl. Cancer Inst.*, 88:908-917, 1996; Landstrom et al., *Int. J. Cancer*, 67:573-579, 1996). Details of how estrogens and antiestrogens elicit these responses has remained unclear.

## SUMMARY

The invention is based on the discovery that: (1) normal human prostate epithelial cells (PreCs) and certain prostate cancer cells express exclusively ER $\beta$ . (2) estrogen/ anti-estrogen action in normal and malignant prostate epithelial cells is mediated by ER $\beta$ . and (3) anti-estrogen-induced cell growth inhibition (cell death/apoptosis/cell cycle arrest) in prostate cancer cells is mediated through an ER $\beta$  signaling mechanism. In addition, there are seven genes in prostatic cancer cells that display ER $\beta$ -mediated up-regulation in response to the antiestrogen ICI 182,780. Furthermore, ER- $\beta$  is exclusively expressed in the nuclei of basal cells in normal prostate and down regulation of ER $\beta$  expression occurs during prostatic carcinogenesis. Specifically, ER- $\beta$  is not expressed in high-grade dysplasia or in grade 4/5 neoplasms, but is expressed in grade 3 lesions. ER- $\beta$  is also expressed in metastatic lesions.

Based, in part, on these discoveries, the invention features *in vitro* screening methods for identifying a compound that modulates ER $\beta$ -mediated cell growth inhibition. Some of these methods include: (a) providing a mammalian test cell containing a functional ER $\beta$  protein; (b) contacting the test cell with a candidate compound; and (c) detecting an increase or decrease in the expression of an ER $\beta$ -regulated gene in the presence of the candidate compound, compared to the level of expression of the gene in the absence of the candidate compound. Compounds that modulate ER $\beta$ -mediated cell growth inhibition can promote or inhibit this process. In some embodiments, the test cell contains no detectable ER $\alpha$  protein. In some embodiments, the test cell is derived from a prostate cell, a neuronal cell, an ovarian cell, a breast cell, a cardiovascular cell or a bone progenitor cell. In some embodiments, the

test cell is engineered to contain an exogenous ER $\beta$  gene, *i.e.*, an ER $\beta$  transgene. The ER $\beta$ -regulated gene can be, *e.g.*, receptor-like tyrosine kinase (RYK), 5-hydroxytryptamine A1 receptor (E2c), BCL-2 related A1, embryonic growth/differentiation factor, IL-12, TL1309, or IFN- $\alpha/\beta$  receptor. In some embodiments, a reporter gene coding sequence is operably  
5 linked to an ER $\beta$ -regulated promoter or enhancer. A multiplicity of ER $\beta$ -regulated genes can be monitored to detect an increase or decrease in expression, for example, by using an oligonucleotide array or subtractive hybridization technology.

As used herein, "cell growth inhibition" means cell death, apoptosis, or cell cycle arrest.

10 As used herein, "detecting an increase" in expression of a gene means detecting an increase from any baseline level, including zero expression.

As used herein, "detecting a decrease" in expression of a gene means detecting a decrease to any reduced level, including zero expression.

As used herein, "ER $\beta$ -regulated gene" means a naturally occurring ER $\beta$ -regulated  
15 gene or a gene construct containing any coding sequence operably linked to one or more ER $\beta$ -regulated gene expression control elements.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. In case of conflict, the present application, including definitions will control. All  
20 publications, patent applications, patents, and other references cited herein are incorporated by reference in their entirety.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

## 25 DESCRIPTION OF DRAWINGS

FIG. 1 is a table summarizing the results of RT-PCR analyses of ER $\alpha$ , ER $\beta$ , progesterone receptor (PR), pS2, and androgen receptor (AR) mRNAs among normal and malignant prostatic epithelial cells. Total RNAs were extracted and reverse-transcribed. The resultant cDNAs were subjected to PCR analyses under optimized conditions. The amplified  
30 products were run into 2% agarose gel with ethidium bromide. Three individual

experiments were performed. A summary of the data from RT-PCR analyses performed in three individual experiments (+, *detectable level of expression*; -, *undetectable level of expression*).

FIGS. 2A-2D are graphs summarizing data on effects of antiestrogens and estrogens on cell growth of DU145 cells. Cells ( $5 \times 10^3$  cells per well) were plated in triplicate wells onto a 24-well plate. After 24 hours for cell attachment, cells were treated for 4 days with antiestrogens [(A) ICI and (B) 4OH-TAM] or estrogens [(C)  $17\beta$ -estradiol and (D) DES] at various concentrations as indicated. Cells treated with vehicle (absolute ethanol) were used as control. The number of viable cells at the end of 4 days of treatment was determined by the trypan blue exclusion method. Three individual experiments were performed. Columns, means; bars, S.D.;  $n = 9$ ; \*,  $p < 0.001$  compared to control (shaded column).

FIGS. 3A-3D are graphs summarizing data on effects of antiestrogens and estrogens on cell growth of PC-3 cells. Cell growth assay was described in Figure 3. (A) ICI. (B) 4OH-TAM. (C)  $17\beta$ -estradiol. (D) DES. Columns, means; bars, S.D.;  $n = 9$ ; \*,  $p < 0.001$ ; #,  $p < 0.01$ ; +,  $p < 0.05$ ; compared to control (shaded column).

FIG. 4 is a graph summarizing data on reduction of ICI-induced inhibition of DU145 cell growth by ER $\beta$  antisense oligonucleotide (ODN). Cells were treated with ICI at  $10^{-6}$  M for 4 days in the presence of 2.5  $\mu$ M antisense, sense or mismatch ODNs. Columns, means; bars, S.D.;  $n = 16$ ; \*,  $p < 0.001$  compared to control without ODN treatment.

FIG. 5 is a graphic representation of the results of a competitive ELISA assay and illustrates the concentration-dependent competition of the immunizing peptide (ER $\beta$ C) with a GC-17 antibody. Preincubation with 4 mg/ml of the control N-terminus peptide (ER $\beta$ N) and GC-17 failed to compete out the binding to the recombinant ER- $\beta$  peptide, demonstrating the specificity of the antibody. The GC-17 antibodies, bound to recombinant ER- $\beta$  protein on ELISA plates, were recognized by alkaline phosphatase conjugated anti-rabbit IgG antibodies. The whole complexes were visualized by incubation with p-nitrophenyl phosphate. The optical density at 405 nm was measured. The entire assay was done 4 times. The columns represent the standard deviation. w/o = GC-17 preincubated without peptide antigen.

FIGs. 6A and 6B are representations of the structures of various phenyl vinyl substituted estrogens (6A) and a Table (6B) showing the effects of various phenyl vinyl

substituted estrogens (a type of candidate compound) on cell proliferation in DU-145 cells. %Cell# indicates the percentage of cells surviving after treatment with a candidate compound compared to untreated controls. MW is the molecular weight of the candidate compound.

FIGs. 7A and 7B are representations of the amino acid and nucleic acid sequences of human estrogen receptor beta.

### DETAILED DESCRIPTION

Any cultured, ER $\beta$ -containing mammalian cells can be employed as mammalian test cells in new *in vitro* screening methods for identifying compounds that modulate ER $\beta$ -mediated cell growth inhibition. The cells can be in primary cell culture or in an established line such as a PrEC prostate epithelial cell line. An example of an established PrEC cell line useful in the invention is BPH-1 (described in Hayward et al., *In Vitro Cell Dev. Biol. Anim.*, 31:14-24, 1995; DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, cat. # ACC143), a nontumorigenic, SV40-immortalized, highly differentiated human prostate epithelial cell line. Another example of a useful, established prostate epithelial cell line is available commercially as PrEC<sup>TM</sup> (Clonetics). An alternative to using an established cell line is to isolate and maintain highly enriched populations of normal PrECs in primary cultures, for example, as described below, in the "Examples." Also useful as test cells are prostatic cancer cell lines such as LNCap (ATCC no. CRL-1740), PC-3 (ATCC no. CRL-1433), or DU-145 (ATCC no. HTB-81). A useful non-prostate cell line is ovarian cell line SKOV-3.

Since estrogens are involved in the development and maintenance of the neuronal tissues, identification of compounds that reduce (rather than promote) ER $\beta$ -mediated apoptosis in neuronal tissues may be desirable, and can be identified using an *in vitro* screening method of the invention. In some embodiments of the invention designed to identify compounds particularly suitable for neurological use, a test cell of neurological origin is used. Test cells having other origins, e.g., cardiovascular cells or bone progenitor cells, can be employed as appropriate, where identification of compounds to treat particular tissues is sought.

The necessary ER $\beta$  protein in the cell can be expressed from an endogenous gene or an exogenous (engineered) gene. The ER $\beta$  gene can be carried on a transient expression

vector. Preferably, however, an exogenous ER $\beta$  gene is stably integrated, so that repeated transfections are not a necessary part of the assay protocol. In some embodiments of the invention, an engineered ER $\beta$  gene is placed under the control of an inducible promoter. Testing in the presence and absence of the inducer provides a convenient way to confirm that  
5 an observed effect of a candidate compound is ER $\beta$ -mediated. If the effect is ER $\beta$ -mediated, it will be reduced or absent when the test cell is contacted with the candidate compound in the absence of the inducer.

The test cell can contain other natural ER subtypes, *e.g.*, ER $\alpha$ , or genetically engineered ER molecules in addition to ER $\beta$ . In addition, the test cell can contain an  
10 estrogen receptor co-regulator (co-activator or co-repressor). However, a test cell containing exclusively ER $\beta$  (and optionally an estrogen receptor co-regulator) advantageously reduces the probability of "false positives," *i.e.*, candidate compound-dependent gene expression changes that are not ER $\beta$ -mediated. To promote reliability and consistency in screening assay results, levels of one or more ER subtypes in test cell lines can be assayed periodically.  
15 Such assays can be based on ER protein level, mRNA level, or both. ER protein assays and ER mRNA assays can be performed by a person of ordinary skill in the art, using conventional methods and materials.

The ER $\beta$ -regulated gene whose expression is monitored in an *in vitro* screen method of the invention can be any ER $\beta$ -regulated gene in the test cell, *e.g.*, receptor-like tyrosine  
20 kinase (RYK), 5-hydroxytryptamine A1 receptor (E2c), BCL-2 related A1, embryonic growth/differentiation factor, IL-12, TL1309 or IFN- $\alpha$  receptor. Expression of the gene can be monitored at any level, *e.g.*, mRNA or protein.

In some embodiments of the invention, a reporter gene coding sequence is engineered into the ER $\beta$ -regulated gene or driven by an ER $\beta$ -regulated promoter-enhancer such as AP-1,  
25 ERE or electrophilic response element. For example, a fusion protein containing all or part of the ER $\beta$ -regulated gene product and all or part of a reporter gene product could be expressed in the assay. The reporter gene coding sequence is chosen to provide an assay "read-out" that is more convenient or more rapid (or both), relative to an assay read-out based on the native ER $\beta$ -regulated gene. Examples of useful reporter genes include those  
30 that encode a fluorescent protein, *e.g.*, green fluorescent protein (GFP) or a variant thereof; a



bioluminescent protein, e.g., luciferase; a colorimetric enzyme, e.g.,  $\beta$ -galactosidase.

Various reporter genes are known in the art and can be used in gene constructs for assaying expression of the ER $\beta$ -regulated gene. See, e.g., Cubitt et al., 1995, "Understanding improving and using green fluorescent proteins," *Trends in Biochemical Science* 20:488-455.

5 The GFP domain of a fusion protein can be encoded by a mammalian-optimized version of a GFP cDNA. See, e.g., Cormack et al., 1997, *Microbiology* 143:303-311.

An *in vitro* screening method of the invention can involve detecting an increase or decrease in the expression of a multiplicity of ER $\beta$ -regulated genes, rather than a single ER $\beta$ -regulated gene. This can be accomplished, for example, by contacting the test cell's RNA  
10 with an oligonucleotide array (see, e.g., Behling et al., 2001, *Proc. Natl. Acad. Sci., USA*, 98:1176-1181; and Hawthorne et al., 2000, *Circulation*, 102:3046-3052, 2000) or by employing subtractive hybridization technology.

In addition to being useful for screening synthetic compounds for potential use as pharmaceutical agents, the methods of the invention can be used to test dietary components,  
15 e.g., red wine, for the presence of estrogen mimics that trigger ER $\beta$ -mediated processes, thereby affecting physiological function positively or negatively. Similarly, the methods of the invention can be used to test environmental pollutants for the presence of estrogen mimics that may pose health risks involving ER $\beta$ -mediated processes.

Various compounds can be screened using the new methods. The ligand binding  
20 cavity of ER $\beta$  is different from that of ER $\alpha$  and therefore it will be possible to develop receptor-specific ligands that may form the basis of novel pharmaceuticals with better *in vivo* efficacy and side effect profile than currently available drugs to target disease cells that express ER- $\beta$  only. Several broad classes of compounds can be screened. Useful compounds include those that specifically bind to ER $\beta$  or are predicted to bind to ER $\beta$ . Compounds that  
25 specifically bind to ER $\beta$  and do not have significant binding to ER $\alpha$  are also useful. Such compounds are identified using methods known in the art. Other useful compounds are those known to have estrogen or anti-estrogen activities in one or more tissues or cell types (e.g., prostate or nervous system).

Other compounds that can be screened include ER- $\beta$  agonists such as anti-estrogen  
30 therapeutics (tamoxifen, ICI 164,384, ICI 182,780 (see, e.g., Van Den Bemd et al., 1999, *Biochem. Biophys. Res. Commun.*, 261(1):1-5; PMID: 10405313), and diethylstilbestrol),

xenocompounds such as environmental pollutants and industrial waste, dental compounds (2',3',4', 5'-tetrachlorobiphenyl-ol, bisphenol A, 4-tert-octylphenol, phytoestrogens (compounds present in the plants, mostly as part of our diet), Quercetin, genistein, resveratrol, and natural estrogens and metabolites (e.g., estradiol, and 17 epiestriol). ER- $\beta$  antagonists can also be used. Examples include: 5,11-cis-diethyl-5,6,11, 12-tetrahydrochrysene-2,8-diol (see, Meyers et al., 1999, *J. Med. Chem.*, 42(13):2456-68 (PMID: 10395487) and Sun et al., 1999, *Endocrinology*, 140(2):800-4 (PMID: 9927308).

Based on the test results described herein, prostate carcinogenesis is characterized by a loss of ER $\beta$  expression at the protein and transcript levels in high grade dysplasia, a reappearance of ER $\beta$  expression in grade 3 cancers, and the diminution/ absence of the receptor in grade 4/5 neoplasms. ER $\beta$  is also expressed in most bone and lymph metastasis of prostate cancer. Therefore, in some embodiments of the invention a prostate dysplasia or cancer cell is assayed for ER $\beta$ . This assay is useful for diagnosis (e.g., staging) of prostate cancer, determining the prognosis for an individual who has prostate cancer, and determining an appropriate treatment for an individual who has prostate cancer.

One method of assaying ER $\beta$  is using an antibody that specifically binds the receptor. Methods of generating such an antibody are described herein.

### EXAMPLES

The following examples are meant to illustrate, but not limit the invention.

#### **Example 1: Establishment of Normal Prostate Epithelial Cells in Primary Cultures**

Tissue specimens used for generating primary cultures of normal Prostate Epithelial Cells in Primary Cultures (PrECs) were obtained from patients undergoing transrectal ultrasound-guided biopsies of the prostate for standard clinical indications. All patients contributing biopsy material were invited to participate in a prospective tissue acquisition study approved by the local IRB. Written informed consent was obtained prior to biopsy from participating patients. From each participant, one biopsy core was obtained from the peripheral zone of the prostate, placed on a sponge pad soaked in sterile saline, a 1-2 mm section was excised from the mid-portion of the core, and suspended in 5 ml of culture medium (described below). The two remaining ends of the core were inked at the ends opposite the sectioned mid-portion piece, placed in 10% formalin, and processed for

histology. Histological examination of the end pieces of a biopsy core allowed us to determine the histologic nature and the homogeneity of the core. Only specimens judged to be histologically normal, with no hyperplastic or neoplastic tissue contamination, were used to establish primary cultures of normal PrECs.

5 Each harvested tissue specimen was then washed three times with Hank's balanced salt solution (HBSS) and cut into 5-7 smaller pieces. The pieces were suspended in 2ml freshly prepared growth medium (see below) and transferred to a 60-mm Falcon culture dish (Becton Dickinson, Lincoln Park, NJ) coated with Type I rat tail collagen (Collaborative Biomedical Products, Bedford, MA). An epithelial cell selection medium (the growth  
10 medium) was used to obtain enriched populations of PrECs. The epithelial cell selection medium was essentially as described in Bright et al., *Cancer Res.*, 57:995-1002, 1997, consisting of: keratinocyte serum-free medium with 25 µg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 2 mM L-glutamine, 10 mM HEPES buffer, P/S (100 U/ml Penicillin and 100 µg/ml Streptomycin), 5.5 µl/ml fungizone, 20 ng/ml cholera toxin and 1%  
15 heat-inactivated fetal bovine serum (FBS). All culture reagents were obtained from Life Technologies (Grand Island, NY) except for FBS, which was purchased from Sigma (St. Louis, MO). The culture was incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere without disturbance for 7 days to allow epithelial cells to grow out of the tissue pieces. Culture medium was then routinely replaced every 4 days until cell culture reached approximately  
20 80% confluence. The cells were split once before they were used for RNA extraction.

In addition to primary cultures obtained from biopsy explants, a batch of normal human prostate epithelial cells (PrEC™) were purchased from Clonetics Co. (San Diego, CA). The PrEC™ cells were cultured in the PrEGM medium supplemented with SingleQuots™ (Clonetics Co., San Diego, CA) according to the manufacturer's  
25 recommended protocol.

#### **Example 2: Maintenance of Established Prostatic Cell Lines**

All culture reagents were obtained from Life Technologies (Grand Island, NY) and FCS from Sigma (St. Louis, MO) except otherwise specified. BPH-1 (described in Hayward  
30 et al., *In Vitro Cell Dev. Biol. Anim.*, 31:14-24, 1995), a nontumorigenic, SV40-immortalized, highly differentiated human prostate epithelial cell line was provided by Dr.

Simon Hayward, Univ. of California at San Francisco. This cell line was maintained in RPMI1640 with 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 4.5 g glucose/L and 1.5 g sodium bicarbonate/L (ATCC, Rockville, MD) plus 5% heat-inactivated FBS, ITS+<sup>TM</sup> (Insulin-Transferrin-Selenium mixture, Collaborative Biomedical Research, Bedford, MA), and P/S. Three human prostate cancer cell lines (DU145, PC-3 and LNCaP) were purchased from ATCC. For routine maintenance, DU145 and PC-3 cells were grown in DMEM/F-12 supplemented with heat inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 M non-essential amino acids, P/S, 0.05 mM  $\beta$ -mercaptoethanol (Sigma Co), and 1% ITS+<sup>TM</sup>. LNCaP were maintained in the same medium used for BPH-1 except that ITS+<sup>TM</sup> was left out from the medium. All cell cultures were incubated at 37°C under a 5% CO<sub>2</sub> atmosphere.

### Example 3: RNA Isolation and Reverse Transcriptase-PCR (RT-PCR)

Total cellular RNA was isolated using RNA Stat-60 reagent (Tel-Test Inc., Friendwood, TX) according to protocols provided by the manufacturer. The quality of each total RNA sample was checked and controlled by the following steps: 1) measurement of optical density, 2) running of a denaturing RNA gel capable of detecting possible RNA degradation, as judged by the integrity and intensity of the 18S and the 28S ribosomal RNA signals, and 3) conducting a semi-quantitative RT-PCR for the 18S ribosomal RNA at low cycle numbers. One  $\mu$ g of total cellular RNA was reverse-transcribed using the GeneAmp RNA PCR kit (Perkin-Elmer, CT) and 2  $\mu$ l of the resulting cDNA was used in each PCR.

Intron-spanning primers were either obtained from published literature or designed using the Primer3 Output program (<http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>). Primer sequences for GAPDH (GenBank Acc. No. M33197), ER $\alpha$  (GenBank Acc. No. M12674), ER $\beta$  (GenBank Acc. No. AF051427, see FIG. 7A which illustrates an amino acid sequence of ER $\beta$ , and FIG. 7B, which illustrates an mRNA nucleic acid sequence for ER $\beta$ ), PR (GenBank Acc. No. M1576), and AR (GenBank Acc. No. L29496) are given in Table 1 (below). All PCR conditions were optimized for quantification of relative message contents under non-saturating conditions. Preliminary experiments were conducted to ensure linearity for all semiquantitative procedures. Hot start PCR using AmpliTaq Gold DNA polymerase (Perkin-Elmer, CT) was employed in all amplification

reactions. The enzyme was activated by pre-heating the reaction mixtures at 95°C for six minutes prior to PCR. This protocol was chosen to minimize non-specific product amplification. The routine PCR program was 30 cycles of 1 minutes at 94°C, 1 minutes at 60°C (annealing temperature) and 1 minute at 72°C with the following modifications: (1) amplification for ER $\beta$  cDNA used an annealing temperature of 58°C, (2) amplifications of ER $\alpha$  cDNA and AR cDNA were carried out at an annealing temperature of 55°C. (3) cycle-number for ER $\alpha$  cDNA amplification was set at 35, and (4) GAPDH cDNA was amplified at 26 cycles. GAPDH cDNA levels served as a loading control. Amplification of the correct sequence was verified by direct DNA sequencing of each PCR product from at least two different samples.

#### Example 4: Cell Treatments

##### Treatment of DU145, PC-3 and LNCaP cells with demethylating agents

The three prostatic cancer cell lines were seeded at a density of  $10^4$  cells per ml medium in 25 cm<sup>2</sup> culture flasks, allowed to attach during a 24 hour period, and exposed to two demethylating agents separately. The demethylating agents were added daily in aqueous solution. 5'-azacytidine was added at final concentrations of 2.5  $\mu$ M and 5  $\mu$ M and 5'-aza-2'-deoxycytidine at 0.5  $\mu$ M and 0.75  $\mu$ M, respectively. Culture medium was changed every four days and cells were subjected to a total of 8 days of demethylating agent treatment. At the end of the treatment period, the medium was removed, and cellular RNA extracted for RT-PCR.

##### Treatment of DU145 and PC-3 cells with estrogens/antiestrogens

Cells were seeded at a density of  $5 \times 10^3$  per ml into 24-well plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) in a final volume of 1 ml culture medium with 5% charcoal-stripped FBS. Twenty-four hours following seeding, triplicate wells of cells were treated in with 1  $\mu$ M, 10  $\mu$ M and 100  $\mu$ M of estradiol-17 $\beta$  (E<sub>2</sub>), diethylstilbestrol (DES), 4'-hydroxytamoxifen (4OH-TAM) or ICI 182,780 (ICI). E<sub>2</sub>, DES, and 4OH-TAM were purchased from Sigma Co. (St. Louis, MO) and ICI was a gift from Zeneca Pharmaceuticals (Macclesfield, United Kingdom). Estrogens and antiestrogens were dissolved in absolute ethanol (Sigma Co., St. Louis, MO) and added to the media daily. Cell cultures not treated

with estrogenic compounds received absolute ethanol as a vehicle control. Total additive ethanol concentrations never exceeded 0.2% throughout the culture period. The cells were re-fed with freshly prepared medium every other day. At the end of a 4-day treatment period, cells in each well were trypsinized and cell count determined by direct counting using the Trypan blue exclusion method. All treatment experiments were repeated at least three times to generate statistically relevant data.

Treatment of DU145 cells with ICI and ER $\beta$  antisense oligonucleotide (ODN)

DU145 cells ( $5 \times 10^3$  cells per well) were plated in 24 well-plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). After allowing 24 hours for cell attachment, cell cultures were treated in triplicate with  $1 \mu\text{M}$  ICI in the presence of  $2.5 \mu\text{M}$  ER $\beta$  antisense sense or mismatch ODNs for 4 days. The ER $\beta$  antisense ODN, an 18-mer, was designed to recognize the first translation start site on the ER $\beta$  mRNA and its immediate 5' flanking region (Table 2, below). The nucleotide sequence of sense ODN is complementary to those of ER $\beta$  antisense ODN (Table 2). Based on the sequence of ER $\beta$  antisense ODN, 5 nucleotides were scrambled to generate a mismatch ODN that retains the same GC ratio of the ER $\beta$  antisense ODN (Table 2). Both the sense and the mismatch ODNs served as controls for the antisense ODN. In all three ODNs, the first and the last 3 nucleotides were phosphorothioate-modified to increase their stability *in cellulo*. Number of viable cells in each well was determined by direct counting using the Trypan blue exclusion method after a 4-day treatment period. At least three individual experiments were performed to obtain statistically relevant data.

Statistical analysis was performed by using Student SYSTAT software (Course Technology, Inc., Cambridge, MA). Data was analyzed by one-way ANOVA followed by the Tukey post-hoc test and a 95% confidence limit was used for all comparisons among treatment groups.

**Example 5: AR, ER $\beta$ , PR and pS2 mRNA Expression**

Five primary cultures of normal PrECs (N4#6, N3#5, N3#4, N2#3, N2#2) were established from ultrasound-guided peripheral zone biopsies over a period of 18 months. Upon histological examination, the biopsy cores were all judged to contain only normal

prostatic tissue with no BPH or cancerous foci contamination. The primary cell cultures were all early passages (second or third), cobblestone in appearance, with no visible fibroblast contamination. Semiquantitative RT-PCR analyses (FIG. 1) demonstrated that normal PrEC cultures retained high levels of androgen receptor (AR) mRNA expression, which usually disappeared in late passage normal PrEC primary cultures or in established PrEC. All five cultures of normal PrECs expressed uniform levels of ER $\beta$  RNA, and transcripts of the estrogen responsive genes, PR and pS2. In contrast, expression of ER $\alpha$  mRNA was noticeably absent in all five cultures even when high cycle number PCR (>42 cycles) was used to amplify the cDNA. PrEC<sup>TM</sup> (Clonetics Co), a commercially prepared normal PrEC culture, and BPH-1, a SV-40 immortalized prostatic epithelial cell line, expressed both ER $\alpha$  and ER $\beta$ , but no PR or pS2 transcripts in PrEC<sup>TM</sup> and only minimal level of PR mRNA in BPH-1.

All three prostatic cancer cell lines, DU145, PC-3 and LNCaP, express ER $\beta$  mRNA (FIG. 1). In contrast, ER $\alpha$  mRNA was expressed only in the PC-3 cells. Interestingly, PR transcripts were detected only in DU145 and LNCaP cells, and not in PC-3 cells. Messages of pS2 were found in PC-3 and LNCaP cells, but not in DU145 cells. In accordance with reports in the literature, AR mRNA expression was only noted in LNCaP cells.

When RT-PCR analyses were conducted for ER $\alpha$  mRNA semiquantification in PrEC<sup>TM</sup> (Clonetics Co), BPH-1 or PC-3 cells we noticed that, in addition to the expected PCR product, a smaller PCR product was co-amplified. Sequencing analysis revealed that this smaller PCR product was derived from an ER $\alpha$  mRNA variant which had whole exon 2 deleted.

Prior to exposure to demethylating agents, ER $\alpha$  and pS2 transcripts were not detected in RNA samples prepared from DU145 cells (FIG. 1). After the 8-day treatment with 5'-aza-cytidine (2.5 $\mu$ M and 5 $\mu$ M) or 5'-aza-2'deoxyctidine (0.5 $\mu$ M and 0.75 $\mu$ M), DU145 cells regained expression of both transcripts. Interestingly, the absence of AR mRNA expression in DU145 cells was not reversed by treatment with demethylating agents. Exposure of LNCaP cells to demethylating agents also reactivated ER $\alpha$  mRNA expression.

**Example 6: Effect of Anti-estrogens and Estrogens on Cell Growth of DU145 and PC-3**

Cell growth analyses showed that the growth of DU145 cells, which only expressed ER $\beta$  mRNA, was adversely affected by the antiestrogens, ICI and 4OH-TAM (FIGs. 2A and 2B). A dose-dependent inhibition of cell numbers was observed in cultures exposed to ICI for 4 days when compared to control cultures treated with vehicle (absolute ethanol). A 40% reduction ( $p < 0.001$ ) in the cell numbers was achieved with an ICI dose of 1 $\mu$ M. A similar growth inhibitory response was observed when DU145 cells were treated with 4OH-TAM. However, cell number reduction achieved with 1 $\mu$ M of 4OH-TAM was only around 25% ( $p < 0.001$ ). In contrast, exposure of DU145 cells to estrogens (E2 and DES) did not affect cell growth in 4-day exposure experiments (FIGs. 2C and 2D).

When PC-3 cells, which expressed transcripts of both ER subtypes, were exposed to antiestrogens (ICI and 4OH-TAM) a 25-30% reduction in cell growth was noted in cultures treated with 1 $\mu$ M or 10 $\mu$ M of ICI, or with 1 $\mu$ M of 4OH-TAM ( $p < 0.001$ , FIGs. 3A and 3B). Furthermore, exposure of PC-3 cells to E2 at 1 $\mu$ M or 10 $\mu$ M concentrations also induced inhibition of cell growth ( $p < 0.05$  and  $p < 0.01$ , respectively; FIG. 3C). Treatment with DES at the various concentrations did not elicit statistically significant cell growth inhibition in PC-3 cells (FIG. 3D).

These data demonstrate that proliferation of prostate cancer cells can be decreased by treatment with ER $\beta$  ligands, e.g., an antiestrogen. The data also show that cell growth assays can be used to identify compounds.

#### **Example 7: Reduction of ICI-Induced Cell Growth Inhibition by ER $\beta$ Antisense ODN**

DU145 cells when treated with ICI at 1 $\mu$ M induced a 40% reduction in cell number (FIG. 4, first column). Co-treatment of DU145 cells with ICI and an ER $\beta$  antisense ODN led to restoration of cell number ( $p < 0.001$ , Fig. 4, second column) while co-treatments with an ER $\beta$  sense ODN or a mismatch ODN (Table 2) did not reverse the ICI-induced effects (FIG. 4, third and fourth columns). These data indicated that the ICI-induced cell growth inhibition (cell death/apoptosis/cell cycle arrest) in DU145 cells is mediated through an ER $\beta$  signaling mechanism.

This is a method of testing whether the effects of a candidate compound are mediated by ER $\beta$ .



Table 1: Primer Sequences for RT-PCR Analysis

Target gene	Primer sequence	Location	Expected Size
ER $\beta$	ER $\beta$ 1:5'TGA AAA GGA AGG TTA GTG GGA ACC3' (SEQ ID NO: 1)	nt.230-253	528 bp
	ER $\beta$ 2:5'TGG TCA GGG ACA TCA TCA TGG3' (SEQ ID NO: 2)	nt.737-757	
ER $\alpha$	ER $\alpha$ 1:5'TAC TGC ATC AGA TCC AAG GG3' (SEQ ID NO: 3)	nt.41-60	650 bp
	ER $\alpha$ 2:5'ATC AAT GGT GCA CTG GTT GG3' (SEQ ID NO: 4)	nt.671-690	
PR	PR-1:5'GAT TCA GAA GCC AGC CAG AG3' (SEQ ID NO: 5)	nt.1817-1836	533 bp
	PR-2:5'TGC CTC TCG CCT AGT TGA TT3' (SEQ ID NO: 6)	nt.2330-2349	
pS2	PS2-1:5'GGA GAA CAA GGT GAT CTG CG3' (SEQ ID NO: 7)	nt.52-71	236bp236bp
	PS2-2:5'CAC ACT CCT CTT CTG GAG GG3' (SEQ ID NO: 8)	nt.268-287	
AR	AR-1:5'CTC TCT CAA GAG TTT GGA TGG CT3' (SEQ ID NO: 9)	nt.2896-2918	342 bp
	AR-2:5'CAC TTG CAC AGA GAT GAT CTC TGC3' (SEQ ID NO: 10)	nt.3214-3237	
GAPDH	GAPDH-F:5'CCA CCC ATG GCA AAT TCC ATG GCA3' (SEQ ID NO: 11)	nt.152-175	598 bp
	GAPDH-R:5'TCT AGA CGG CAG GTC AGG TCC ACC3' (SEQ ID NO: 12)	nt.726-749	

Table 2: Oligonucleotide Sequences for Antisense ODN Experiments

Target gene	Sequences	Location <sup>a</sup>	Size
ER $\beta$			
Antisense	5'C <sup>*</sup> A <sup>*</sup> T <sup>*</sup> CAC AGC AGG GCT	-15 to +3	18 bp
Sense	A <sup>*</sup> T <sup>*</sup> A <sup>*</sup> 3' (SEQ ID NO: 13)		
Mismatch	5'T <sup>*</sup> A <sup>*</sup> T <sup>*</sup> AGC CCT GCT GTG		
	A <sup>*</sup> T <sup>*</sup> G <sup>*</sup> 3' (SEQ ID NO: 14)		
	5' <u>G</u> <sup>*</sup> A <sup>*</sup> T <sup>*</sup> <u>CTC</u> AGC <u>ACG</u> GCA		
	A <sup>*</sup> A <sup>*</sup> T <sup>*</sup> 3' (SEQ ID NO: 15)		

\* This base was phosphorothioate-modified.

Ⓐ The first base of translation-initiating site is +1.

### Example 8: ER $\beta$ Modulation of Gene Expression

cDNA array technology was used to identify genes whose expression is modulated by ICI 182,780 in DU145 cells. The DU-145 cells were treated for 12 hours with ICI 182,780. and total RNA was then isolated from treated cells and untreated controls. Following poly-(A)-RNA selection of these two population of total RNA, and DNase I digestion to remove contaminating DNA, the poly (A)-RNA pools were reversed-transcribed in the presence of P32 to produce radiolabeled cDNA probes. These radiolabeled cDNA probes were applied to an Atlas<sup>TM</sup> cDNA Expression Array (600 gene array, Clontech Laboratories, Inc. Palo Alto CA) using the vendor's recommended protocol. Seven genes were identified as being up-regulated in response to cell treatment with ICI 182,780. The seven up-regulated genes are listed in Table 3 (below).

**Table 3**  
**ER $\beta$  Regulated Genes**

Name of Gene (Position on Array)	Fold difference relative to control	Potential functions
RYK receptor-like tyrosine kinase (E2c)	+3.6	The human ryk tyrosine kinase found to represent a ubiquitously expressed gene. Message localization found in epithelial and stromal compartment of tissues such as brain, lung, colon, kidney, and breast. Increased expression in ovarian tumors, and pediatric brain tumors, an anaplastic ependymoma, a glioblastoma multiforme and a primitive neuroectodermal tumor
5-hydroxytryptamine A1 receptor (E4h)	+6.6	It belongs to the G protein coupled serotonin (5-hydroxytryptamine) receptor subtype. It plays a role in mediating the neurotrophic effects of serotonin. Expression is detected in midbrain dorsal raphe nucleus and activation of the receptor inhibits the firing of serotonin neurons and the release of neurotransmitter. In astrocytes it play a role in the synaptic plasticity necessary for certain experience-driven brain changes, such as memory or learning. It modulates fear-related behavior.

BCL-2 related A1 protein (BFL-1)(C4H)	+9.6	BFL-1 is an anti-apoptotic member of the BCL-2 family. The bfl-1 protein suppresses apoptosis induced by the p53 tumor suppressor protein in a manner similar to other Bcl-2 family such as Bcl-2, Bcl-xL and EBV-BHRF1. Activation of monocytes leads to dramatic induction of Bfl-1. It is abundantly expressed in bone marrow. In tumor tissues, its expression was preferentially detected in infiltrating inflammatory cells rather than in cancer cells.
Embryonic growth/differentiation factor (GDF-1)	+4.5	GDF-1 is a member of the transforming growth factor beta superfamily that is expressed in the brain. In mice GDF-1 is most likely an extracellular factor mediating cell differentiation events during embryonic development.
Interleukin-12 alpha chain (natural killer cell stimulatory factor, p35) (IL-12)(F6K)	+10.6	IL-12 promotes cell-mediated immunity by facilitating type 1 helper T-lymphocyte responses, inducing the secretion of interferon-gamma from both T and natural killer cells, enhancing the lytic activity of natural killer cells, and augmenting specific cytolytic T-lymphocyte responses. IL-12 plays an important role in normal host defense against infection by a variety of intracellular pathogens. Inhibition of IL-12 synthesis may be beneficial in diseases associated with pathologic Th1 responses, such as multiple sclerosis or Crohn's disease.. It may also have clinical utility in the treatment of patients suffering from chronic hepatitis B or C virus infections.
TL1309	+2.7	
IFN- $\alpha/\beta$ receptor	+2.7	

Genes regulated by ER $\beta$  can be useful e.g., as secondary targets for treating prostate cancer. For example, up-regulation of ER $\beta$ -induced genes could be a useful method of regulating prostate cell proliferation.

- 5           There are additional reports in the literature of cell lines expressing only ER- $\beta$  that include genes regulated by this receptor. Examples include the thymidylate synthase gene, survivin gene, telomerase gene, hTERT gene, a subunit of the enzyme telomerase, tumor necrosis factor-alpha (TNF-alpha) and quinone reductase. See, e.g., Nakayama et al., "Tamoxifen and gonadal steroids inhibit colon cancer growth in association with inhibition of thymidylate synthase, survivin and telomerase expression through estrogen receptor beta mediated system," *Cancer Lett.*, 2000, 161(1):63-71 (PMID: 11078914); Routledge et al., "Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and Er $\beta$ ," *J Biol Chem.*, 2000, 275(46):35986-93 (PMID: 10964929); Misiti et al., "Induction of hTERT expression and telomerase activity by estrogens in human ovary epithelium cells," *Mol. Cell. Biol.*, 2000 20(11):3764-71 (PMID: 10805720); An et al., "Estradiol repression of tumor necrosis factor-alpha transcription requires estrogen receptor activation function-2 and is enhanced by coactivators," *Proc. Natl. Acad. Sci., USA* 1999,
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96(26):15161-6 (PMID: 10611355); Srivastava et al., "Estrogen decreases TNF gene expression by blocking JNK activity and the resulting production of c-Jun and JunD," *J. Clin. Invest.*, 1999, 104(4):503-13 (PMID: 10449442).

5 **Example 9: Identification of Additional ER $\beta$  Modulated Genes**

The above nucleotide array experiments are repeated using ICI 182,780 and higher density arrays to identify additional ER $\beta$ -modulated genes. Such arrays are obtained commercially from sources including New England Nuclear (Boston, MA)( $>2000$  gene array); Research Genetics (Huntsville, AL)( $> 5000$  gene array); and Genome Systems Inc.  
10 (See Incyte Genomics, Palo Alto, CA)( $> 7,000$  gene array). Some of the additional ER $\beta$ -modulated genes are up-regulated, e.g., about 2-fold to about 50-fold. In contrast, some of the additional ER $\beta$ -modulated genes are down-regulated, e.g., about 2-fold to about 50-fold. The relationship of these genes to the ER $\beta$  and apoptosis/cell death/cell cycle arrest pathway is confirmed by analysis of RNA pools isolated from: (1) cells treated with ICI 182,780 and  
15 not treated with ER $\beta$  antisense oligonucleotide; and (2) cells treated with ICI 182,780 and ER $\beta$  antisense oligonucleotide. Genes regulated (or induced) by ICI 182,780 acting via ER $\beta$  show reduced response to ICI 182,780 in the presence of the ER $\beta$  antisense oligomer.

The following examples describe the generation of an ER $\beta$ -specific antibody, and illustrate that prostatic carcinogenesis was characterized by a loss of ER $\beta$  expression at the  
20 protein and transcript levels in high-grade dysplasias, its reappearance in grade 3 cancers, and its diminution/absence in grade 4/5 neoplasms.

**Example 10: Generation of an ER $\beta$ -Specific Polyclonal Antibody (GC-17)**

To make an ER $\beta$ -specific antibody, an immunizing peptide was selected with aid of  
25 the computer programs Protean (DNASTAR, Inc., Madison, WI) and Peptool (BioTools, Inc., Edmonton, AB, Canada). A peptide sequence in the F domain of the human ER $\beta$  receptor (amino acids 449-465) was selected, as there is no homology with estrogen receptor alpha (ER $\alpha$ ) at this region (Mosselman et al., *FEBS, Lett* 392:49-53, 1996; Gustafsson JA, *Semin Perinatol*, 24:66-69, 2000). The peptide was custom synthesized by Research Genetics  
30 (Huntsville, AL) with a format of 4-branch Multiple Antigenic Peptide. Each rabbit (male NZW, 5-6 lbs) was first inoculated with 0.5 mg peptide antigen with complete Freund's

adjuvant, and then boosted with 0.25 mg peptide plus Incomplete Freund's adjuvant at day 14, day 21, and every two weeks afterward until a satisfactory serum titer was obtained. A direct Enzyme-Linked ImmunoSorbent Assay (ELISA) was used to assess the immune responses to the peptide antigen (Harlow et al., *A Laboratory Manual*, 139-242, 1988).

5

#### **Example 11: Methods Used to Test the Specificity of the GC-17 Antibody**

##### *Competitive inhibition ELISA assay*

The wells of an ELISA plate, PRO-BIND<sup>™</sup> (Becton-Dickenson Labware, Franklin, NJ) were coated with a recombinant protein composed of the entire ER $\beta$  sequence (PanVera, Madison, WI) at a concentration of 5 $\mu$ g/ml. The GC17 antibody (1:6000) was then pre-  
10 incubated with the immunizing peptide at concentrations ranging from 4mg-4 $\mu$ g/ml at room temperature for 30 minutes. In addition, 4 mg of a control peptide encompassing sequences in the N-terminal region of ER- $\beta$  (Research Genetics) was preincubated with the GC17 antibody (1:6000) at room temperature for 1 hour. The resulting antigen/antibody complexes  
15 were then incubated with the bound recombinant ER- $\beta$  protein on the ELISA plate at 37°C for 2 hours. Alkaline phosphatase conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) was used to recognize the GC17 antibody, which bound to recombinant ER $\beta$  protein on the plate. The whole complexes were visualized by incubation with p-Nitrophenyl phosphate in 2-amino, 2-methyl, 1,3-propanediol buffer pH  
20 9.6. Results were quantified by optical density using the Microplate reader 550 (Bio-Rad, Richmond, CA). The entire assay was done four times.

##### *Competitive Immunohistochemistry*

Lau et al. (*Cancer Res* 60:3175-3182, 2000) have recently demonstrated that DU145  
25 and LNCaP cells, both derived from a metastatic prostate cancer, express abundant ER $\beta$  mRNA but not ER $\alpha$  message. These cells were used to compliment and confirm that the GC-17 antibody reagent specifically detected ER $\beta$  but not ER $\alpha$  by immunohistochemical staining. Using the GC-17 antibody and the anti-ER $\alpha$  antibody (NCL-ER-6F11, Novacastra, Newcastle, UK) at the same dilutions as for tissue sections (see below), we carried out  
30 immunohistochemical studies on 10% formalin-fixed cytopins of DU145 and LNCaP cells

that had been routinely processed, embedded in paraffin, sectioned at 5  $\mu$ m and mounted on SuperFrost Plus™ slides (VWF Scientific, West Chester PA).

Peptide competition studies were performed at the immunohistochemical level that approximated the conditions used in the ELISA assays described above. GC-17 antibody, at a dilution of 1:6000, was incubated with the immunizing ER $\beta$  peptide at concentrations of 400 $\mu$ g and 40 $\mu$ g at room temperature for 1 hour. In addition, competitive studies were conducted using ER $\alpha$  recombinant peptide (400 and 40 $\mu$ g, Affinity Bioreagents Inc., Golden, CO) on DU145 cells. Incubation conditions and time were identical to those used for the ER $\beta$  peptide competition studies. Deparaffinized sections of DU145 and LNCaP cells and human prostate tissue were then incubated with these mixtures at room temperature for 1 hour. Competition studies, done on prostate tissues, were identical to those performed on DU145 cells, except the peptide and antibody mixtures were incubated overnight and then applied to sections for 24 hours at room temperature. All of the remaining immunohistochemical and other staining procedures were identical to those used for tissue sections (see immunohistochemical procedures).

#### *Western Blot Analysis*

Four human normal prostate tissues from radical prostatectomies and one normal human testis tissue were used in this analysis. In addition, we used normal prostate epithelial cells (Clonetics, walkersville, MD) and DU145 cells (ATCC, Rockville, MD) for these studies. Recombinant proteins, ER $\alpha$  (RP310) and short form of ER- $\beta$  (RP311) (Affinity Bioreagents Inc., Golden, CO) as well as long form of ER- $\beta$  (PanVera, Madison, WI), were included as controls. Tissues or cells were homogenized in radioimmunoprecipitation (RIPA) buffer containing 50mM Tris-HCl pH 7.4, 1% Nonidet P-40 (Amresco, Solon, OH), 0.5% sodium deoxycholate, 0.1% SDS, 1mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol, 1mM activated sodium orthovanadate and 2X Complete™ proteinase inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Twenty-five  $\mu$ g of tissue protein extracts, 0.5  $\mu$ g of recombinant ER $\alpha$  protein or 0.5  $\mu$ g of recombinant ER $\beta$  protein were mixed with 2X SDS loading buffer (125mM Tris buffer pH 6.8, 20% glycerol, 2% SDS, 2%  $\beta$ -mercaptoethanol and 1 $\mu$ g/ml bromophenol blue) and electrophoresized onto a 10% SDS-polyacrylamide gel under reducing condition. The separated proteins were transferred onto a PolyScreen® PVDF transfer membrane (NEN, Boston, MA). The membrane was incubated

for 1 hour in blocking buffer (PBS with 5% nonfat dry milk). The primary antibodies were applied at 1:6000 for GC17 ER- $\beta$  antibody or 1:5 for 1D5 ER $\alpha$  antibody (Biogenex, Mountainview, California) in PBS-T (PBS with 0.05% Tween-20) buffer with 0.1% bovine serum albumin for overnight at room temperature. After washing 5 times with PBS-T buffer, the membrane was incubated with horseradish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for GC17 or goat anti-mouse IgG antibody (NEN, Boston, MA) for 1D5 at 1:2500 for 1 hour. The signals were visualized with chemiluminescence ECL detection system (NEN, Boston, MA) and autoradiography. All reagents were purchased from Sigma (St. Louis, MO) unless specified.

#### Example 12: Preparation of Prostate Tissues

##### *a) Formalin fixed radical prostatectomy specimens:*

Tissues studied were from 50 radical prostatectomy specimens collected at Stanford University Medical School, during the years 1995 -1999. Patients ranged in age from 46-73 years of age and none had received any treatment prior to their undergoing prostatectomy. Prostates were fixed in 10% buffered formalin for 24 hours then sectioned transversely. Tissues were dissected fixed in 10% buffered formalin for 3 hours, routinely processed, and embedded in paraffin. A histopathological diagnosis was made on a hematoxylin and eosin stained (H&E). The criteria used in the grading of the carcinomas were those described by Stamey et al., *JAMA*, 281:1395-1400, 1999. The slide, together with the corresponding paraffin block, was then sent to IL where immunohistochemical studies were carried out. At least one section from each case was stained with H&E and reviewed by IL to assure that it matched the tissue components in the original slide. Paraffin sections were cut at 6  $\mu$ m mounted on SuperFrost™ Plus slides. Sections were left unbaked until used for immunohistochemical studies.

Among the 50 cases selected for study, 26 contained areas of carcinoma. Five of these were clear cell carcinomas of the transition zone while all the remaining cancers were found in the peripheral zone. All of the peripheral zone cancers were composed of mixtures of grade-3 and 4/5 carcinomas. In contrast, all of the clear cell carcinomas were predominately grade-3 neoplasms. Twenty of the peripheral cancer specimens also contained varying amounts of low/moderate to high-grade dysplastic lesions. Dysplasia of the

peripheral zone was found in the absence of carcinoma in 6 of the 50 total cases we studied. Additionally, 7 of the 50 cases. were low/moderate grade dysplasias of the central zone that did not coexist with cancer. Among the 50 cases. two specimens each of lesion-free normal peripheral, central and the transition zone were included in our study. Among the cases  
5 studied, 15 examples of benign prostatic hyperplasia (BPH) were either commingled with other lesions (Ho et al.. *CRC Press*. 73-113. 1997) or occurred separately (Leav et al.. *AM J Pathol*, 93:69-92, 1978).

*b) Bone and lymph node metastases:*

In addition to the prostatectomy cases. archived paraffin blocks containing bone  
10 metastases were obtained from 7 patients, treated at University of Massachusetts Medical Center. The patient's ages ranged from 59-74 and they were all clinically stage D2 at the time of diagnosis. All received antiandrogen treatment as follows: 1) Four patients were orcheotomized. One of these patients was given the LH/RH agonist Lupron (TAP Pharmaceuticals inc. Deerfield IL) and the AR competitive inhibitor Eulixin (Flutamide-  
15 Schering corp. Kenilworth. NJ) for 3 months. one treated with Eulixin for 24 months and the remaining two were not given any further antiandrogenic therapy. 2) Three patients were not orcheotomized. Two were treated with Lupron for 8 months and the other with Lupron and Eulixin for 3 months. Following these antiandrogenic therapies for the periods noted above, it was determined that all seven patients were failing therapy. At those time points.  
20 biopsies of suspected bone metastases were obtained from the iliac crest of each patient. These samples were immediately fixed in 10% buffered, routinely processed. embedded in paraffin. 6µm sections placed on SuperFrost™ Plus slides, and stained with H&E. Replicate sections of these lesions were used for immunohistochemical studies.

In addition, we also studied 5 archived examples of metastases to regional lymph  
25 nodes. Two cases were obtained from Department of Pathology at University of Massachusetts Medical School and the remaining 3 were from University of Florida Medical School (a gift from Dr William Murphy). The patients were 60-85 years of age. Regional lymph nodes (external iliac and pelvic) were obtained from all patients during radical prostatectomy. Only one patient had received any treatment prior to surgery (Lupron).

*c) Frozen tissues for LCM/RT-PCR*



Eighteen separate specimens, derived from radical prostatectomies, were placed in cassettes containing TBS (Triangle Biomedical Sciences, Durham, NC) and quick-frozen in liquid nitrogen. Approximately fifteen minutes elapsed from the surgical removal of the gland to the initiation of freezing. Formalin-fixed and paraffin embedded tissue sections, immediately adjacent to the quick frozen specimens, were also taken for subsequent immunohistochemical studies (see below).

For diagnostic purposes and lesion selection, tissues were first cryostat sectioned and then fixed briefly in 70% ethanol and stained with H&E. The frozen tissue blocks were then stored at -70°C until they were used for microdissection. Prior to LCM, sections from these cases were found to contain varying amounts of grade three and 4/5 carcinoma as well as dysplastic and normal glands.

#### Example 13: Immunohistochemical Procedures

The followings are the primary antibodies and the dilutions used in our studies:

Anti-estrogen receptor beta (ER $\beta$ ), rabbit polyclonal antibody GC-17, diluted at 1:6000; anti-estrogen receptor alpha (ER $\alpha$ ), mouse monoclonal antibody NCL-ER-6F11, diluted at 1:50 (Novocastra, Newcastle upon Tyne, UK); anti-androgen receptor (AR), rabbit polyclonal antibody, diluted to 22.7 $\mu$ g/ml (Upstate Biotechnologies, Lake Placid, NY); anti-Mib5/Ki67, mouse monoclonal antibody, diluted at 1:50 (Immunotech, Westbrook, ME) and anti-high molecular weight cytokeratin (HMWC), mouse monoclonal antibody 34 $\beta$ E12, diluted at 1:50 (Enzo Diagnostics, Farmingdale, NY). Immunostaining for Prostatic Specific Antigen (PSA) was done with a Nexus Immunostainer (Ventana, Tuscon Ariz.) using prediluted reagents.

Five  $\mu$ m thick sections were cut and mounted on SuperFrost<sup>TM</sup> Plus slides. Sections were left unbaked until immediately prior to use at which point they were baked for 1 hour at 60°C. After baking, sections were deparaffinized through three changes of xylene and rehydrated through graded alcohols into water. Heat induced epitope retrieval (HIER) was performed by boiling sections in citrate buffer pH 6.0 (pH 6.2 for ER $\beta$ ) for 15 minutes on a laboratory hotplate. After boiling, sections were removed from the hotplate, allowed to cool at room temperature (RT) for 20 minutes, and were then rinsed thoroughly with water (sections stained for PSA did not require HIER). Sections were then placed in 3% hydrogen

peroxide for 15 minutes at RT to block endogenous peroxidase. washed with water. and placed in PBS (Sigma. St.Louis. Mo). Sections were then incubated with Power Block (Biogenex) nonspecific blocking reagent for 10 minutes at RT to reduce nonspecific staining. washed with water. and placed in PBS. Sections were then incubated with normal goat serum at 1:50 (Vector, Burlingame, CA) for 15 minutes at RT. The goat serum was then shaken off and sections were incubated with primary antibodies overnight at 4°C. After overnight incubation, each section received 20 seconds of washing with PBS, 20 seconds of washing with Biogenex Optimax Detergent Wash Solution followed by 10 minutes of washing in PBS on a rotator. Solutions were changed for every eight slides. Following washing. sections were incubated with either Biogenex Multilink secondary antibody at a dilution of 1:20 for 20 minutes at RT or DAKO (Carpinteria. CA) ready to use secondary antibody for 10 minutes at RT. Sections were again washed according to the protocol described above. Sections were then incubated with either Biogenex Streptavidin-conjugated horseradish peroxidase at a dilution of 1:20 for 20 minutes at RT or DAKO ready to use streptavidin-conjugated alkaline phosphatase for 10 minutes at RT. Sections were again washed as previously described. Immunostaining was visualized using either Biogenex liquid 3,3'-diaminobenzidine (Biogenex) or DAKOs New Fuchsin as the chromogen. Following development, sections were rinsed in water. lightly counterstained with 10% Harris Modified Hematoxylin.

Positive controls for GC-17 included DU145 cells and tissue sections of prostate. which were previously shown to be consistently stained with the antibody. Positive tissue controls for ER $\alpha$  were human breast cancers, shown to contain numerous immunostained cells. Morphologically normal human prostate sections served as positive controls for AR as well as for HMWC and MIB5/Ki-67 stains. For all reagents, negative controls were performed by substituting the primary antibody with a class-matched isotype.

#### **Example 14: Laser capture microdissection (LCM) and RT-PCR**

In all instances, immunohistochemical studies for ER- $\beta$  were performed on the paraffin tissue sections, which are adjacent to the frozen sections used for microdissection and RT-PCR analysis. Frozen sections were cut on a cryostat at 5 $\mu$ m placed on precleaned glass slides (Fisher Scientific, Pittsburgh PA) and immediately fixed in 70% ethanol for 5

seconds. The sections were then briefly dipped in distilled water, stained with 10% Harris hematoxylin for 15 seconds, dipped in distilled water, then successively placed in 70% ethanol for 30 seconds, briefly immersed in 1% eosin then placed in 95% ethanol 1 minute, two changes of 100% ethanol 1 minutes each, and two changes of xylene 30 seconds each.

5 After air-drying for approximately 30 minutes tissues were microdissected using a Pixcell 2 Laser microdissection unit (Arcturus, Mountainview, CA). Eight to ten normal acini were microdissected from each of three different cases. Similarly, 10-20 dysplastic glands were dissected from 4 separate cases of high-grade lesions, and approximately the same numbers of neoplastic glands were obtained from 5 cases of grade 3 and six different examples of

10 grade 4/5 carcinomas.

RNA was extracted from each sample and then separately subjected to RT-PCR analysis. Total cellular RNA was separately isolated using RNA Stat-60 reagent (Tel-Test Inc., Friendwood, TX) according to protocols provided by the manufacturer. The total isolated cellular RNA was reverse-transcribed using the GeneAmp RNA PCR kit (Perkin

15 Elmer, CT) in total 20 $\mu$ l reaction mixture and 2 $\mu$ l of the resulting cDNA was used in PCR on ER- $\alpha$ , AR and GAPDH and 3 $\mu$ l for PCR on ER- $\beta$ . Hot start PCR using AmpliTaq Gold DNA polymerase (Perkin-Elmer, CT) was employed in all amplification reactions. The enzyme was activated by pre-heating the reaction mixtures at 95°C for 6 minutes prior to PCR. The PCR programs were 45 cycles for GAPDH and 55 cycles for ER- $\alpha$ , AR and ER- $\beta$

20 of 1 minutes at 94°C, 1 minute at 60°C (annealing temperature) and 1 minute at 72°C. This protocol was chosen to minimize non-specific product amplification.

The primer sequences for ER $\alpha$ , AR and GAPDH were described in our previously study (Lau et al., *Cancer Res*, 60:3175-3182, 2000). The primer set for ER $\beta$  was newly designed and the forward primer is 5'-GATGAGGGGAAATGCGTAGA-3' (SEQ ID NO:16)

25 and the reverse primer is 5'-CTTGTTACTCGCATGCCTGA-3' (SEQ ID NO:17).

#### **Example 15: Specificity of the GC-17 antibody**

##### *Competitive ELISA*

Pre-incubation of GC-17, an antibody raised against the F domain of ER $\beta$ , with the

30 immunizing peptide (C-terminus of ER $\beta$ , ER $\beta$ C) successfully suppressed binding to the recombinant protein FIG. 5. The suppression occurred in a concentration-dependent manner

when compared to the control where the antibody was not pre-incubated with the immunizing peptide. In contrast, pre-incubation of GC-17 with the control N-terminus peptide of ER $\beta$  (ER $\beta$ N; 4 mg/ml) did not significantly suppress binding when compared with the control, indicating that the antibody was not cross-reactive with this region of the ER $\beta$  protein.

#### *Competitive immunochemistry*

Strong nuclear immunostaining was detected in sections of DU145 and LNCaP cells which served as positive controls for the peptide competition studies. Pre-incubation of GC-17 with either 400  $\mu$ g/ml or 40  $\mu$ g/ml of the immunizing peptide ERBC totally abolished nuclear staining in sections of these cells, when compared with positive controls where the peptide was omitted. Identical results were obtained with sections of human. Preincubation of GC17 with the recombinant ER $\alpha$  protein failed to block ER $\beta$  immunostaining of DU145 cells by the antibody. These studies confirmed that GC-17 does not cross react with ER $\alpha$  and supports data from the Western Blot findings (infra). Thus, both the competitive ELISA and competitive immunohistochemistry showed GC-17 to be highly specific for binding to the ER $\beta$  protein.

In addition, DU145 and LNCaP cells, that only express ER $\beta$ , Lau et al. (*Cancer Res* 60:3175-3182, 2000), were negative when immunostained with the ER $\alpha$  (NCL-ER-6F11) antibody. Positive staining of prostate tissues with the ER $\alpha$  antibody was restricted to stromal cells (see immunohistochemistry of normal prostate below).

#### *Western Blot Analysis*

Using Western blot analysis, GC-17 was demonstrated to specifically recognize two recombinant ER $\beta$  proteins and show no cross-reactivity to ER $\alpha$  protein. The size of recombinant ER $\beta$  protein (RP311) from Affinity Bioreagents Inc. (Golden, CO) is approximately 53kDa and it represents a short form of ER- $\beta$  protein using a reported initiator codon (corresponding to 43-530 Kuiper et al., *Proc Natl Acad Sci USA*. 93:5925-5930.1996; Mosselman et al., *FEBS Letter*, 392:49-53. 1996; Tremblay et al., *Mol Endocrinol*. 11:353-365. 1997 amino acids). The long form of ER $\beta$  recombinant protein (PanVera, Madison, WI), containing 530 amino acids, was also recognized by GC-17 antibody and showed a 59kDa band in the blot. The native long form of ER $\beta$  protein in the cells is approximately

63kDa. GC17 recognized an approximately 63kDa protein in human normal testis and prostate tissues, suggesting that the long form may be the natural ER $\beta$  protein in human tissues. The size difference between long form of recombinant ER- $\beta$  protein and native protein in cells may be related to the occurrence of post-translational modifications in cells and tissues. The level of ER $\alpha$  protein in human normal testis and prostate tissues was undetectable with the 1D5 human antibody after a 30s exposure to X-ray film. However, very weak ER $\alpha$  protein signal was detected only when the blot was exposed to an X-ray film for over 10 minutes.

#### Example 16: Immunohistochemistry of prostate tissues

##### *Normal prostate*

In morphologically normal ducts and acini, nuclear ER $\beta$  expression was consistently densely localized in nuclei of basal cells as defined by HMWC staining in replicate sections). Strong nuclear staining was absent in secretory cells but frequently observed in stromal cells. Occasionally nuclear membrane staining for the receptor was also evident in a few luminal cells. ER $\alpha$  immunostaining was not present in secretory cells of normal ducts and acini but individual scattered receptor positive basal cells were observed in less than 10% of all sections studied. The ER $\alpha$  receptor was, however, consistently found in stromal cell nuclei, especially in periglandular locations.

Pronounced nuclear staining for AR was a constant finding in secretory and stromal cell nuclei. In agreement with a past study (Gustafsson et al., Semin Perinatol, 24: 66-69, 2000), variable immunostaining for AR was also observed in individual basal cells of normal glands. No difference was found in the cellular localization of the three steroid hormone receptors when the peripheral, transition, and central zones of the prostate were compared. Moreover, the same localization of the steroid hormone receptors, found in the three normal zones, was also seen in foci of BPH.

The most consistent immunolocalization for the three receptors in basal cells was found within periurethral ducts.

##### *Dysplastic lesions*

Immunohistochemical findings in dysplastic and carcinomatous lesions are summarized in Table 4.

**Table 4: Immunohistochemical finding in dysplasias and carcinomas**

Lesion	ER- $\alpha$	ER- $\beta$	AR
<u>Dysplasia/ Peripheral zone</u>			
Moderate grade*	-	+	+
High grade	-	-	+
<u>Dysplasia/Central zone*</u>	+	+	+
<u>Carcinoma/ Peripheral zone</u>			
Grade3	+/+	+	+
Grade 4/5	+	+/+	+
<u>Carcinoma/ Transition zone</u>	-	-	+
<u>Metastatic carcinoma</u>			
Bone	-	+	+
Lymph nodes	+/++	+	+

\*Data reflects positive staining in dysplastic and basal cells. Staining restricted to residual basal cells in high-grade dysplasia. "+" = Positive staining in > 50% of cases; "-/++" = Positive staining in 40% of cases; "-/++" = Positive staining in < 20% of cases. See text for estimates of the % of positively stained cells in each lesion.

In the peripheral zone, a consistent pattern of ER $\beta$  expression was found in dysplastic lesions. A secretory cell localization for nuclear ER $\beta$  expression was commonly observed in low to moderate grade dysplastic lesions. The majority of both basal and dysplastic secretory cells in these lesions, contained moderate to strongly stained nuclei. A marked diminution to total absence of ER $\beta$  immunostained nuclei was a consistent feature in almost all dysplastic cells in the high grade lesions that were studied. Staining was however present in residual basal cells within these high-grade lesions. Thus, a loss of ER- $\beta$  staining in high-grade dysplasias paralleled a decline in receptor-positive basal cells. In contrast to normal cells, the cytoplasm of dysplastic cells in lesions of all grades but especially in high grade lesions were frequently stained by the GC-17 reagent, a feature not seen when the primary antibody was omitted from the incubation or with the use of any of the other antireceptor reagents. Similarly nuclear membrane of cells in high grade dysplastic cells also frequently

stained with the GC-17 antibody. ER- $\alpha$  positive dysplastic cells were not present within any of the peripheral zone lesions that were studied.

In marked contrast, ER- $\alpha$  stained cells were detected in 5 of 7 (71%) of dysplasias in the central zone. There was great variation in the numbers (10-90%) of immunopositive cells in any given central zone lesion. In replicate sections, 6 of these lesions contained dysplastic cells that were also positive for ER- $\beta$  staining.

As previously reported (Leav et al., *Prostate*, 29:137-145, 1996), AR was strongly expressed in the majority (>95%) of dysplastic cells irrespective of the origin or grade of the lesion.

#### *Grade 3 and 4/5 carcinomas*

A transition from ER- $\beta$  positive to negative staining of cells was observed in all 21 cases where cancer was found in the peripheral zone that paralleled the progression of the grade 3 carcinomas to the less differentiated grade 4/5 neoplasms. ER $\beta$  positive cells were found in 13 of 15 (87%) grade 3 carcinomas of the peripheral zone. The spectrum of expression ranged from examples where all nuclei in an individual neoplastic gland were strongly stained to instances where receptor immunostaining was weak and/or found in few cells within a given microscopic field. The latter examples were most often located in areas where a transition to higher-grade carcinoma occurred. Unlike their counterparts in the peripheral zone, the vast majority of cells comprising grade 3 clear cell carcinomas in the transition zone were devoid of ER $\beta$  immunostaining. In two of five cases, scattered receptor positive neoplastic cells were found in a minority of glands.

An almost complete absence of ER $\beta$  nuclear staining was seen in all but 3 of 15 (20%) grade 4/5 carcinomas. In the majority of cases however staining of the nuclear membrane was frequently apparent. In the 3 cases, positive cells represented only 10% or less of the total cancer cells in a given lesion and the staining intensity was usually diminished.

Immunostaining for ER $\alpha$  was detected in only two (7.6%) of the total twenty-six cases of primary carcinoma we studied. In these two instances, a few (< 10%) weakly positive cells were found in both grades 3 and 4/5 carcinomas. Staining for the receptor was consistently absent in all clear cell carcinomas of the transition zone.

Irrespective of grade, strong nuclear AR immunostaining was a constant feature in the vast majority cells (>95%) comprising cancers of the peripheral zone. Nuclear AR immunostaining was also present in almost all grade 3 clear cell carcinomas but it was less intense than found in peripheral zone carcinomas. Interestingly, the occasional few cells that were ER- $\beta$  positive in these cancers were found to be negative for AR expression in replicate sections.

No change in the location or intensity of immunostaining for the three receptors in the stroma was evident in sections that contained carcinoma.

#### *Metastatic lesions*

Nuclear ER $\beta$  immunostaining was present in metastatic carcinoma cells in bone lesions from all but one of the seven cases. The intensity of signal did vary from strong to weak staining within cells comprising the lesions of individual case and/or among the cases. In three instances, carcinoma cells were surrounded by a prominent desmoplastic response in which ER $\beta$  staining was frequently found in the nuclei of fibroblasts. Nucleated hematopoietic marrow cells were also positive for the receptor while mature red cells were negative, a finding that served as a positive and negative internal tissue control for ER $\beta$  immunostaining in these lesions.

In contrast to the finding of ER $\beta$  immunostaining in foci of all but one case of bone metastasis, no staining was observed for ER $\alpha$  in these lesions. Despite the fact that all of seven patients, including the one that lacked ER $\beta$  expression had been given antiandrogenic therapy, nuclear AR staining was observed in most metastatic cells in the bone biopsies. However, AR staining was also consistently present in the cytoplasm as well as in the nucleus of metastatic cells, a finding observed in bone and lymph node lesions from all patients who received antiandrogenic therapy (see below).

PSA immunostaining was found in metastatic bone lesions in four cases but it tended to be scant especially when compared to lesions in lymph nodes from patients who did not receive antiandrogenic therapy (see following discussion).

Among the five cases of lymph node metastases, two contained a majority of neoplastic cells (>50%) that were uniformly strongly stained for ER $\beta$ . In one case, immunostaining for the receptor was absent in the metastatic cells while in the remaining two cases a mix of negative and positively stained neoplastic cells were found. In one of the two



cases, where strong receptor expression was detected, the patient had been treated with the LH/RH agonist Lupron prior to surgery. Lymphocyte nuclei were consistently stained for the receptor and served as an internal positive tissue control in the two cases where no receptor was detected in metastatic cells within the lymph nodes.

5        ER $\alpha$  immunostained carcinoma cells were found in 2 of 5 cases of lymph node metastases. Both patients were untreated prior to surgery. In one of these cases, ER $\beta$  staining was absent in metastatic cells. In one case, numerous ER $\alpha$  positive cells (>50%) were mixed with those in which receptor expression was absent. Very weak staining for ER $\alpha$  was identified in a few cells (<10 %) in the other case.

10        In all five cases, AR immunostaining was present in the vast majority (> 95%) of metastatic cells. Nuclear AR expression was always strong in cancer cells and with the exception of the case where the patient received Lupron, cytoplasmic staining was not present in any of the metastatic cells. Strong PSA immunostaining was present in the cytoplasm of most metastatic cells in all cases including the one where the patient had  
15        received antiandrogenic therapy.

#### Example 17: LCM/RT-PCR Analysis

Findings with RT-PCR analysis for ER $\beta$  mRNA on LCM lesions approximated the results of immunohistochemical studies done on paraffin sections immediately adjacent to the  
20        frozen specimens used for RT-PCR as well as on other cases. ER $\beta$  transcripts were detected in 2 of the 3 samples of normal prostatic acini. In contrast, receptor message was found in only 1 of 4 microdissected samples of high-grade dysplasias. Sixty percent of grade 3 carcinomas contained ER $\beta$  transcripts while receptor message was detected in 2/6 (30 %) of the grade 4/5 cancers. In two cases where ER $\beta$  message was present in grade 3 lesions,  
25        grade 4/5 carcinomas sampled within the same section, lacked receptor mRNA expression. In close agreement with our immunohistochemical findings, AR mRNA was present in all normal glands, dysplasias, grade 3 cancers, and all but one of the grade 4/5 carcinomas. ER $\alpha$  transcripts were not detected in any of the microdissected specimens that were studied.

The data demonstrate that a consistent pattern of lost ER $\beta$  expression at both the  
30        transcriptional and translational levels occurs during prostatic carcinogenesis and tumor progression. Thus, there may be loss of an important role that the receptor would normally

play in inhibiting growth of the prostate which contributes to neoplastic development. The continued expression of AR throughout these processes, as well as other undefined factors. may therefore exert persistent unopposed growth stimulus acting on these cells.

5     **Example 18: Cytotoxicity Studies with Phenyl Vinyl Substituted Estrogens in DU-145 Cells**

Candidate compounds can be tested for their ability to modulate cell proliferation in cells that express functional ER $\beta$ . The following illustrates this testing method using DU-145 cells.

10     DU-145 cells were treated for 4 days in media containing 1 mM of each of the above reagents after pre-seeding for 2 days in 10 % FBS. Various concentrations of Resveratrol® and ICI-182780 were used as positive controls to confirm previous results, i.e., that these compounds lead to a decrease in cell number of 50-70% at 1 mM respectively under the same culture conditions. The percent cell number after treatment was normalized to cells treated with carrier only. FIG. 6A shows the compounds used, and FIG. 6B shows the results of an experiment in which 17 $\alpha$ -substituted (e.g., phenyl vinyl substituted) estrogens were used in the assay. These data show that the various compounds listed in FIG. 6B range in their ability to inhibit cell proliferation. FIG. 6A shows the structural formulas of the 17 $\alpha$ -E (or Z)-(x-phenyl)-vinyl-E<sub>2</sub>, and the structural formula for X. The chart in FIG. 6B shows the results for the 25 different compounds in which the R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub> groups of X are varied. and the E or Z form is designated.

20     Information can be obtained from such assays regarding not only the ability of the tested candidate compounds to modulate cell proliferation, but also information that is useful for designing additional candidate compounds. For example, examination of the data in FIG. 6B shows that there is a general trend for fluorine labeled compounds to have increased efficacy as inhibitors of cell proliferation in those compounds where the fluorine substitutes are "in close proximity" to the 17 $\beta$ -hydroxy group.

25     A dose response curve can also be generated using an assay such as the cell proliferation assay described above. Flow cytometry (e.g., analysis of DNA density, alexin V/lactate dehydrogenase) can be used to discern pro-apoptotic from cell static mechanisms of

30

inhibition of cell proliferation. Candidate compounds can also be tested in a quinone reductase assay to assess antioxidant response induced gene expression.

Raloxifene® and Resveratrol® may also be active tumor suppressing agents utilizing the ER $\beta$  pathways. Using DU145 and PC-3 M cells that express only ER $\beta$ , we found that Raloxifene, an antiestrogen marketed as Evistar by Eli Lilly, and Resveratrol, a phenolic compound found in grape skin and seeds, produced marked inhibition of cell growth. The efficacy of Raloxifene is markedly better than that of Resveratrol and ICI. Treatment protocols and dosages used were similar to those described above.

#### Example 19: ER $\alpha$ and ER $\beta$ Expression in Normal and Neoplastic Testicular Tissue

Estrogen exposure has been a postulated etiologic factor in the development of testicular neoplasms, and epidemiologic studies have associated prenatal estrogen exposure with an increased risk for germ cell cancer. To date, however, estrogen receptors have not been identified in germ cell neoplasms. The recent identification of the novel estrogen receptor (ER) beta prompted us to investigate if it is present in germ cell malignancies and thus might facilitate the suspected carcinogenic effects of estrogens.

Thirty-two archived surgical specimens from men undergoing radical orchiectomy for testicular cancer were evaluated for the presence of ER $\beta$ . Five normal specimens were also examined. All specimens were examined by immunohistochemical stains with both the highly specific anti-human estrogen receptor beta (ER $\beta$ ) antibody (as described *supra*) and with commercially available ER alpha (ER $\alpha$ ) antibody.

In normal testes, ER $\beta$  was localized to the nucleus and highly expressed in spermatogonia and spermatocytes. ER $\alpha$  was not expressed in any testicular specimen, but was noted in adjoining epididymis in two samples. Ninety percent of the seminomas had high expression of ER $\beta$ , the remaining specimen (10%) demonstrated low expression. Forty-two percent of MGCTs with embryonal elements had high expression of ER $\beta$ , whereas 58% had low expression. All five MGCTs with yolk sac elements and all three cases with teratomatous elements had high expression of ER $\beta$ . Both intratubular germ cell neoplasia specimens demonstrated high expression of ER $\beta$ . There was an absence of ER $\alpha$  in both normal and malignant testes.

These data provide the first demonstration of the expression of ER $\beta$  in testicular germ cell tumors. These findings now suggest a potential mechanism for estrogen's putative involvement in germ cell neoplasia. The differential qualitative expression seen in embryonal cells (MGCTs with embryonal elements) shows that assay of ER $\beta$  may be of prognostic significance and be useful for determining the appropriate treatment for certain testicular cancers.

#### OTHER EMBODIMENTS

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. For example, ER $\beta$ -regulated genes can be identified using methods other than oligonucleotide arrays, and ER $\beta$ -regulated genes other than the seven listed above can be used to screen libraries of compounds to identify candidate compounds that modulate ER $\beta$ -mediated cell growth inhibition. Accordingly, other embodiments are within the scope of the following claims.

## 1 WHAT IS CLAIMED IS:

- 1 1. An *in vitro* screening method for identifying a compound that modulates ER $\beta$ -  
2 mediated cell growth inhibition, the method comprising:  
3 (a) providing a mammalian test cell comprising a functional ER $\beta$  protein;  
4 (b) contacting the test cell with a candidate compound;  
5 (c) detecting an increase or decrease in the expression of an ER $\beta$ -regulated gene  
6 in the presence of the candidate compound, compared to the level of expression of  
7 the gene in the absence of the candidate compound, as an indication that the  
8 compound modulates ER $\beta$ -mediated cell growth inhibition.  
9
- 1 2. The method of claim 1, wherein the compound that modulates ER $\beta$ -mediated cell  
2 growth inhibition promotes cell growth inhibition.  
3
- 1 3. The method of claim 1, wherein the compound that modulates ER $\beta$ -mediated cell  
2 growth inhibition inhibits cell growth inhibition.  
3
- 1 4. The method of claim 1, wherein the test cell comprises no detectable ER $\alpha$  protein.  
2
- 1 5. The method of claim 1, wherein the test cell is derived from a cell selected from  
2 the group consisting of a prostate cell, a neuronal cell, an ovarian cell, a breast  
3 cell, a cardiovascular cell and a bone cell progenitor.  
4
- 1 6. The method of claim 5, wherein the test cell is derived from a prostate cell.  
2
- 1 7. The method of claim 1, wherein the test cell contains an exogenous ER $\beta$  gene.  
2
- 1 8. The method of claim 1, wherein the test cell contains an exogenous estrogen  
2 receptor co-regulator.  
3
- 1 9. The method of claim 1, wherein the ER $\beta$ -regulated gene is selected from the  
2 group consisting of receptor-like tyrosine kinase (RYK), 5-hydroxytryptamine A1

3 receptor (E2c), BCL-2 related A1, embryonic growth/differentiation factor, IL-12,  
4 TL1309, and IFN- $\alpha$  receptor.

5  
1 10. The method of claim 1, further comprising a reporter gene coding sequence  
2 operably linked to an ER $\beta$ -regulated gene expression control element.

3  
1 11. The method of claim 1, wherein detecting an increase or decrease in the  
2 expression of an ER $\beta$ -regulated gene is performed using an oligonucleotide array  
3 or a subtractive hybridization technique.

4  
5 12. A method of identifying a compound that modulates ER $\beta$ -mediated cell growth  
6 inhibition, the method comprising comparing the proliferation in a cell  
7 comprising a functional ER $\beta$  protein in the presence and absence of a candidate  
8 compound, wherein a decrease in cell proliferation indicates that the compound  
9 inhibits cell growth inhibition.

10  
11 13. The method of claim 12, wherein the cell does not express ER $\alpha$ .

12  
13 14. A method of identifying a candidate compound that modulates ER $\beta$ -mediated cell  
14 growth inhibition, the method comprising  
15 (a) comparing cell proliferation in a first cell comprising a functional ER $\beta$  protein  
16 in the presence and absence of a candidate compound;  
17 (b) selecting the compound if there is a decrease in cell proliferation in the  
18 presence of the candidate compound; and  
19 (c) comparing cell proliferation in a second cell that does not express functional  
20 ER $\beta$  protein in the presence and absence of the candidate compound, wherein no  
21 difference in cell proliferation in the second cell indicates that the compound  
22 modulates ER-mediated cell growth inhibition.

23  
24 15. The method of claim 14, wherein the second cell is the same cell type as the first  
25 cell.

- 26 16. The method of claim 14, wherein the second cell comprises an antisense ER $\beta$   
27 oligonucleotide.  
28
- 29 17. A compound identified by the method any of claim 1.  
30
- 31 18. A method of determining whether a cell is susceptible to ER $\beta$ -mediated cell  
32 growth inhibition, the method comprising  
33 (a) providing a cell, and  
34 (b) testing the cell for the presence of ER $\beta$ , wherein the presence of ER $\beta$  indicates  
35 that the cell is susceptible to ER $\beta$ -mediated cell growth inhibition.  
36
- 37 19. The method of claim 18, wherein the cell is a cancer cell.

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N4#6	N3#5	N3#4	N2#3	N2#2	PrEC	BPH-1	LNCaP	PC-3	DU145	Sample
-	-	-	-	-	+	+	-	+	-	ER $\alpha$
+	+	+	+	+	+	+	+	+	+	ER $\beta$
+	+	+	+	+	-	-	+	-	+	PR
+	+	+	+	+	-	-	+	+	-	PS2
+	+	+	+	+	+	+	+	-	-	AR

FIG. 1

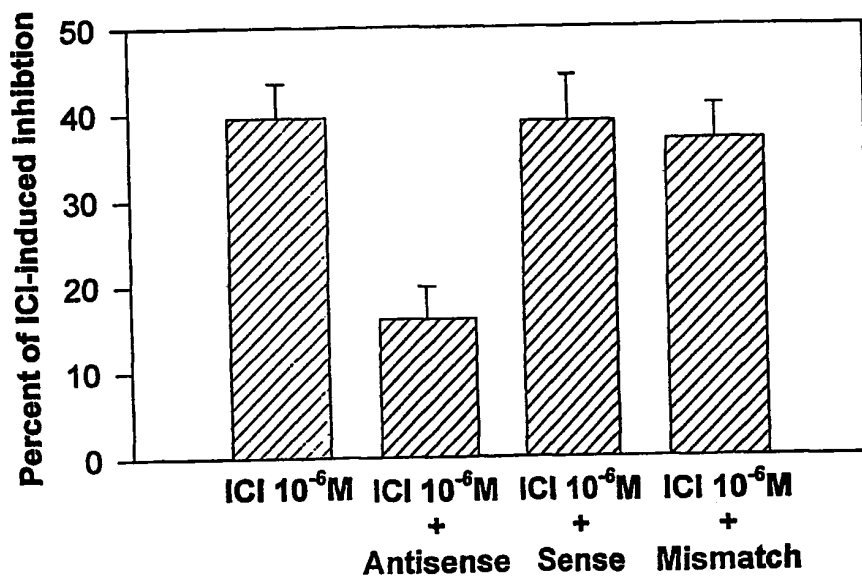


FIG. 4



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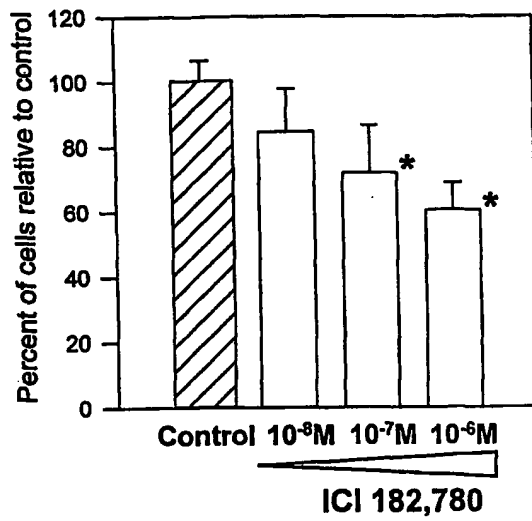


FIG. 2A

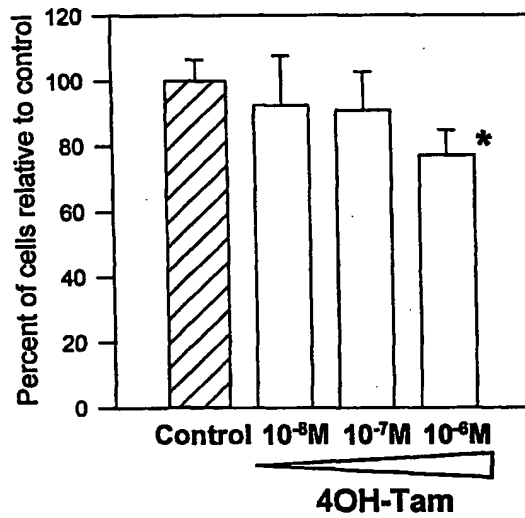


FIG. 2B

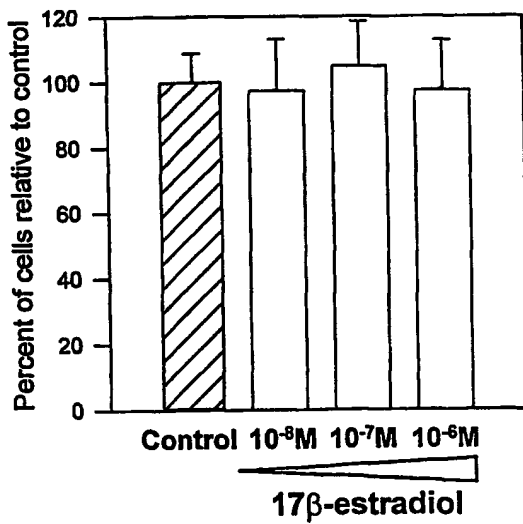


FIG. 2C

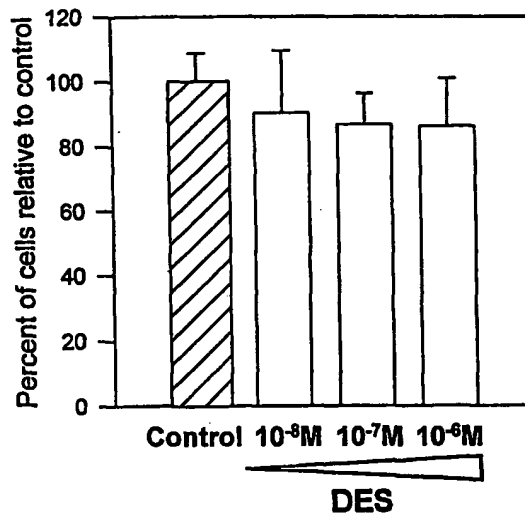


FIG. 2D

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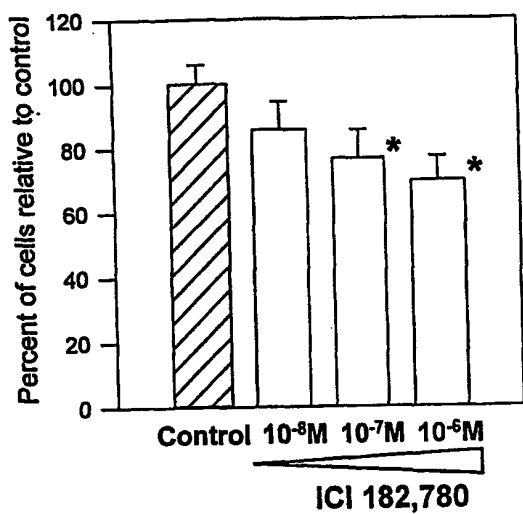


FIG. 3A

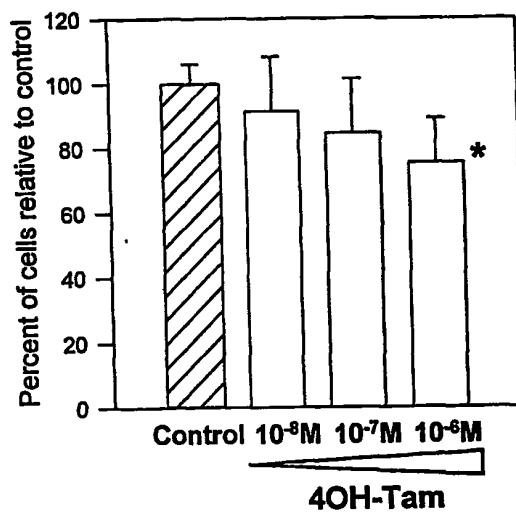


FIG. 3B

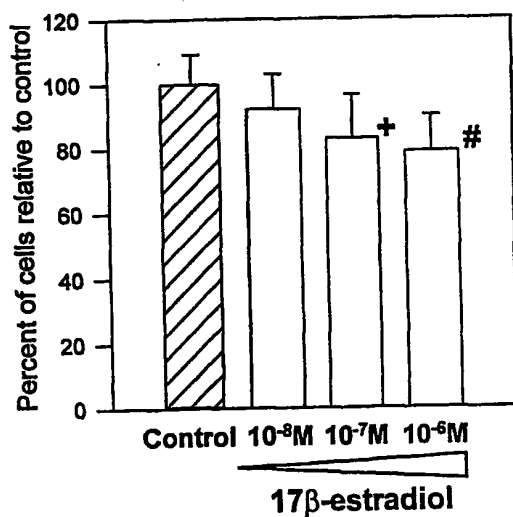


FIG. 3C

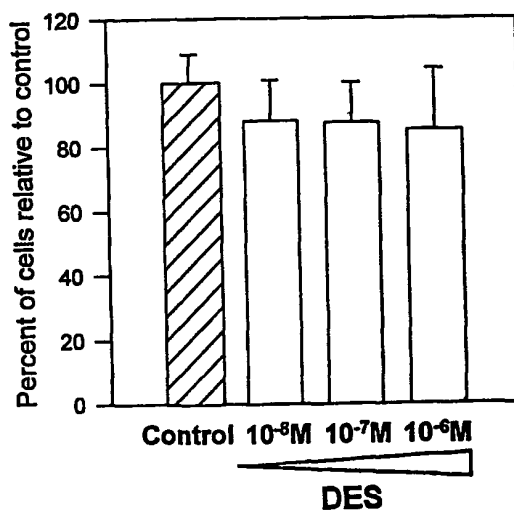


FIG. 3D

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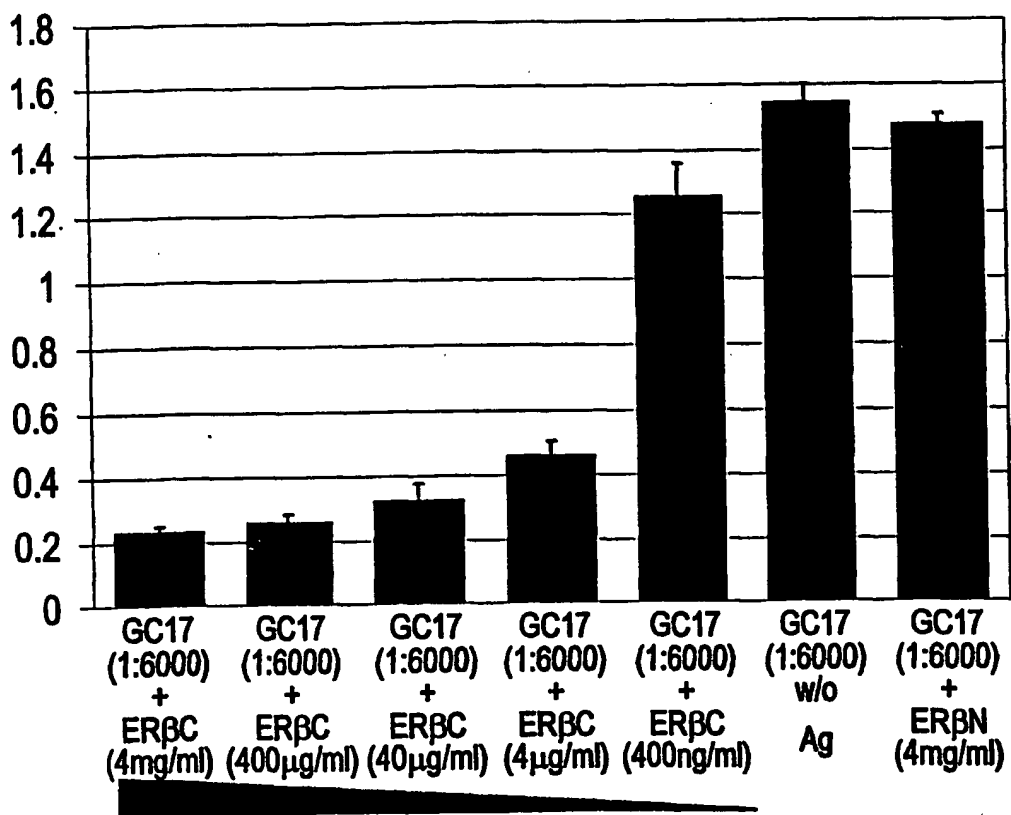
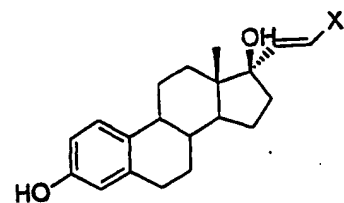
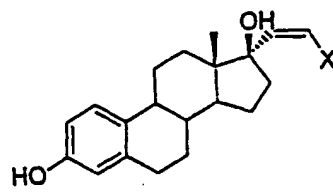


FIG. 5



17α-E-(x-phenyl)-vinyl-E<sub>2</sub>



17α-Z-(x-phenyl)-vinyl-E<sub>2</sub>

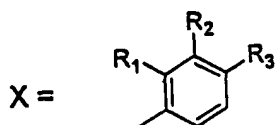


FIG. 6A

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ID #	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	E/Z	mg	%Cell# / SD	MW
1	H	H	H	E	2.0	81/10	374
2	F	H	H	Z	4.6	29/3	392
3	H	H	CH <sub>3</sub>	E	1.3	35/3	388
4	H	H	CO <sub>2</sub> CH <sub>3</sub>	E	1.5	39/1	432
5	H	CH <sub>3</sub>	H	E	2.1	45/5	388
6	H	H	F	Z	2.5	85/3	392
7	H	H	NH <sub>2</sub>	Z	1.2	11/3	389
8	H	H	CO <sub>2</sub> H	E	2.1	63/3	418
9	H	H	F	E	2.0	62/4	392
10	H	H	CN	E	2.5	63/3	402
11	H	H	OCH <sub>3</sub>	Z	1.7	87/6	404
12	CO <sub>2</sub> CH <sub>3</sub>	H	H	E	2.7	85/8	432
13*	-	-	-	?	2.3	36/3	298
14*	-	-	-	-	2.7	50/2	382
15	H	H	CO <sub>2</sub> CH <sub>3</sub>	Z	4.8	52/4	432
16	CH <sub>2</sub> OH	H	H	Z	1.7	62/3	404
17*	-	-	-	Z	2.0	30/2	390
18*	-	-	-	-	3.8	21/2	304
19	CF <sub>3</sub>	H	H	E	1.6	36/2	442
20	CH <sub>2</sub> OH	H	H	E	1.0	43/2	404
21*	-	-	-	E	2.1	79/4	390
22*	-	-	-	E	1.4	55/7	376
23	H	CF <sub>3</sub>	H	E	1.4	53/4	442
24	CO <sub>2</sub> H	H	H	E	2.4	57/2	418
25	H	H	CF <sub>3</sub>	E	1.7	63/4	442

FIG. 6B

• (13) 17 $\alpha$ -vinyl-E<sub>2</sub>; (14) Br-THP-E<sub>2</sub>; (17, 21) E<sub>3</sub>-COC<sub>6</sub>H<sub>5</sub>; (18) THP-E<sub>2</sub>; (22) 17 $\alpha$ -Br-vinyl-E<sub>2</sub>

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...MDIKNSPSSILNSPSSYNCSQSILPLEHGSIIYIPSSYVDSHHEYF  
 AMTFYSPAVMNYISPSNVTNLEGGPGRQTTSPNVLWPTPGHLSPLVVHRQLSHLYAEP  
 QKSPWCEARSLEHTLPVNRETLKRKVSNGRCASPVTGPGSKRDAHFCVCSDYASGYH  
 YGVWSCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVMVKCG  
 SRRERCGYRLVRRORSADQLHCAGKAKRSGGHAPRVRELLLDALSPEQLVLTLEAE  
 PPHVLI SRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLESC  
 WMEVLMGLMWRSIDHPGKLI FAPDLVLDREDEGKCEGILEIFDMLLATTSRFRELKL  
 QHKEYLCVKAMILLNSSMYPLVTATQDADSSRKLALHLLNAVTDALVWVIKSGISSQQ  
 QSMRLANLLMLLSHVRRHASNKGMEHLLNMKCKNVVPVYDLLEMLNAHVLRGCKSSIT  
 GSECSPAEDSKSKEGSQNPQSQ

FIG. 7A

1 tttcagtttc tccagctgct ggcttttttg acacccactc ccccgccagg aggcagttgc  
 61 aagcgcgag gctgcgagaa ataactgcct cttgaaactt gcaggcgcaa gaggcggcg  
 121 cgagcgctgg gccggggagg gaccacccga gctgcgacgg gctctggggc tggggggcag  
 181 ggctggcgcc cggagcctga gctgcaggag gtgcgctcgc tttcctcaac aggtggcgcc  
 241 gggcgcgcg cgggagacc cccctaattg cgggaaaagc acgtgtccgc attttagaga  
 301 aggcgaaggcc ggtgtgttta tctgcaagcc attatacttg cccacgaatc tttgagaaca  
 361 ttataatgac ctttgtgcct cttcttgcaa ggtgttttct cagctgttat ctcaagacat  
 421 ggatataaaa aactcaccat ctgaccttaa ttctccttcc tcctacaact gcagtcaatc  
 481 catcttacc cttggagcacg gctccatata cataccttcc tcctatgtag acagccacca  
 541 tgaatatcca gccatgacat tctatagccc tgctgtgatg aattacagca ttcccagcaa  
 601 tgtcactaac ttggaagggt ggccctggtcg gcagaccaca agcccaaagtg tgttgtggcc  
 661 aacacctggg cacctttctc ctttagtgggt ccatcgccag ttatcacatc tttgtcgga  
 721 acctcaaaag agtccctggt gtgaagcaag atcgctagaa cacaccttac ctgtaaacag  
 781 agagacactg aaaaggaagg ttagtgggaa ccgttgccgc agccctgtta ctggtccagg  
 841 ttcaaagagg gatgctcact tctgcgctgt ctgcagcgat tacgcatcgg gatataacta  
 901 tggagtctgg tcgtgtgaag gatgtaaggc ctttttttaa agaagcattc aaggacataa  
 961 tgattatatt tgtccagcta caaatcagtg tacaatcgat aaaaaccggc gcaagagctg  
 1021 ccaggcctgc cgacttcgga agtggtacga agtgggaatg gtgaagtgtg gctcccggag  
 1081 agagagatgt gggtagcgcc ttgtgcggag acagagaagt gccgacgagc agctgcaatg  
 1141 tgccggcaag gccaaagaaa gtggcgccga cgcgccccga gtgcgggagc tgcgtgtgga  
 1201 cgccctgagc cccgagcagc tagtgctcac cctcctggag gctgagccgc cccatgtgct  
 1261 gatcagccgc cccagtgcgc ccttcaccga ggccctccatg atgatgtccc tgaccaagtt  
 1321 ggccgacaag gaggttggtac acatgatcag ctgggccaag aagattcccg gctttgtgga  
 1381 gctcagcctg ttgcagcaag tgccgctctt ggagagctgt tggatggagg tctaatgat  
 1441 ggggctgatg tggcgctcaa ttgaccaccc cggcaagctc atctttgtct cagatcttgt  
 1501 tctggacagg gatgagggga aatgcgtaga aggaattctg gaaatctttg acatgtcctc  
 1561 ggcaactact tcaagggttc gagagttaa actccaacac aaagaatatc tctgtgtcaa  
 1621 ggccatgatc ctgctcaatt ccagtatgta ccctctggtc acagcgaccc aggatgtgta  
 1681 cagcagccgg aagctggctc acttgctgaa cgccgtgacc gatgctttgg tttgggtgat  
 1741 tgccaagagc ggcattctct cccagcagca atccatgcgc ctggctaacc tctgatgct  
 1801 cctgtccac gtcaggcatg cgagtaacaa gggcatggaa catctgctca acatgaagtg  
 1861 caaaaatgtg gtcccagtg atgacctgct gctggagatg ctgaatgcc acgtgcttcg  
 1921 cgggtgcaag tcctccatca cgggtccga gtgcagcccg gcagaggaca gtaaaagcaa  
 1981 agagggtcc cagaacccac agtctcagtg a

FIG. 7B

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US01/08976

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/74; C12N 15/00

US CL : 435/6; 530/399

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 530/399

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, USPATFULL

search terms: estrogen receptor beta, gene expression, growth inhibition

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KUIPER et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor $\beta$ . Endocrinology. October 1998, Vol. 139, No. 10, pages 4252-4263, especially page 4253.	1, 2, 4-6, 8
X,P	DUPONT et al. Effect of single and compound knockouts of estrogen receptors $\alpha$ (ER $\alpha$ ) and $\beta$ (ER $\beta$ ) on mouse reproductive phenotypes. Development. October 2000, Vol. 127, pages 4227-4291, especially page 4290.	1-11

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Special categories of cited documents		Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed	"A"	document member of the same patent family

Date of the actual completion of the international search

18 JUNE 2001

Date of mailing of the international search report

12 JUL 2001

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ELIANE LAZAR-WESLEY

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US01/08878

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	LAU et al. Expression of estrogen receptor (ER)- $\alpha$ and ER- $\beta$ in normal and malignant prostatic epithelial cells. Regulation by methylation and involvement in growth regulation. Cancer Research. 15 June 2000, Vol. 60, pages 3175-3182, especially Figures 3 and 4.	1-2, 4-6
X,P	NAKAYAMA et al. Tamoxifen and gonadal steroids inhibit colon cancer growth in association with inhibition of thymidylate synthase, survivin and telomerase expression through estrogen receptor beta mediated system. Cancer Letters. 08 December 2000, Vol. 61, No. 1, pages 63-71, especially page 68.	1-2, 4-6
X	LAU et al. Antiestrogens, via ER $\beta$ pathway, inhibit growth of prostate cancer cells. Proc. Am. Assoc. Cancer Res. March 1999, Vol. 40, pages 637-638, see entire abstract #4203.	1-2, 4-6
A	PAECH et al. Differential ligand activation of estrogen receptors ER $\alpha$ and ER $\beta$ at AP1 sites. Science. 05 September 1997, Vol. 277, pages 1508-1510.	1-11

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US01/08976

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-11

Remark on Protest

☐  
☐

- The additional search fees were accompanied by the applicant's protest.  
No protest accompanied the payment of additional search fees.



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US01/08278

**BOX II OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-11, drawn to a method for identifying a compound that modulates ER $\beta$ -mediated cell growth inhibition by detecting a change in the expression of ER $\beta$ -regulated genes.

Group II, claims 12-16, drawn to a method for identifying a compound that modulates ER $\beta$ -mediated cell growth inhibition by measuring cell proliferation.

Group III, claim 17, drawn to a compound.

Group IV, claims 18-19, drawn to a method of testing for the presence of ER $\beta$ .

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature of Group I is the detection of a change in the expression of ER $\beta$ -regulated genes. Groups II-IV do not share the special technical feature of Group I, as Group III is to a compound and Groups II and IV are methods which use different steps and have different end results.

*Handwritten signature/initials*

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